RsmA and AmrZ orchestrate the assembly of all three type VI secretion systems in *Pseudomonas aeruginosa*

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The type VI secretion system (T6SS) is a weapon of bacterial warfare and host cell subversion. The Gram-negative pathogen *Pseudomonas aeruginosa* has three T6SSs involved in colonization, competition, and full virulence. H1-T6SS is a molecular gun firing seven toxins, Tse1–Tse7, challenging survival of other bacteria and helping *P. aeruginosa* to prevail in specific niches. The H1-T6SS characterization was facilitated through studying a *P. aeruginosa* strain lacking the RetS sensor, which has a fully active H1-T6SS, in contrast to the parent. However, study of H2-T6SS and H3-T6SS has been neglected because of a poor understanding of the associated regulatory network. Here we performed a screen to identify H2-T6SS and H3-T6SS regulatory elements and found that the posttranscriptional regulator RsmA imposes a concerted repression on all three T6SS clusters. A higher level of complexity could be observed as we identified a transcriptional regulator, AmrZ, which acts as a negative regulator of H2-T6SS. Overall, although the level of T6SS transcripts is fine-tuned by AmrZ, all T6SS mRNAs are silenced by RsmA. We expanded this concept of global control by RsmA to VgrG spike and T6SS toxin transcripts whose genes are scattered on the chromosome. These observations triggered the characterization of a suite of H2-T6SS toxins and their implication in direct bacterial competition. Our study thus unveils a central mechanism that modulates the deployment of all T6SS weapons that may be simultaneously produced within a single cell.

T6SS | Pseudomonas | RsmA | AmrZ

The type VI secretion system (T6SS) is widely distributed within Gram-negative bacteria and is capable of injecting effector proteins into eukaryotic cells (1). Mounting evidence suggests the primary role of the T6SS is in bacterial warfare (2). The T6SS injects toxins (e.g., peptidoglycan hydrolases) into competing bacteria. Deployment of the T6SS provides a fitness advantage and contributes to shaping bacterial communities.

In about one third of bacterial genomes that harbor T6SS genes, multiple clusters can be found (3). Maintenance of these large operons as well as other specialized T6SS genes (e.g., *vgrG* or *hcp* islands; *SI Appendix*, Fig. S1) (4) suggests all these clusters are functional and give the bacteria a survival advantage. It is thought that multiple clusters are expressed and used in specific conditions, but the regulation or cross-regulation of all T6SS genes within a single bacterium is a topic of scarce knowledge.

The Gram-negative pathogen *Pseudomonas aeruginosa* encodes three T6SS clusters: H1-, H2-, and H3-T6SS (5). H1-T6SS transports at least seven antibacterial toxins (Tse1–Tse7) (6-8). This system has been extensively studied, as it was shown to be active in a *retS* mutant, whereas it is silent in the parental strain (9). This suggested a tight regulation of the T6SS, otherwise poorly expressed in in vitro growth conditions and likely triggered in a specific environment, such as during in vivo colonization. Mutation in the *retS* gene allows activation of the GacS/GacA two-component system, with GacA driving expression of two small noncoding RNAs, RsmY and RsmZ, that sequester RsmA and lead to de-repression of H1-T6SS (9, 10). RsmA is a translational repressor that binds on H1-T6SS messenger RNA (11) immediately upstream of the first gene in the cluster, *tssA1* (12) (*SI Appendix*, Fig. S1). This pathway has been proposed to be required for *P. aeruginosa* to sense kin cell lysis and trigger the "*P. aeruginosa* response to antagonism," which is an increase in H1-T6SS activity and killing of bacterial competitors (13).

Regulators and growth conditions involved in H2- or H3-T6SS expression have been proposed, including quorum sensing and iron limitation (14, 15), but none has a clear effect in vitro compared with RetS or RsmA on H1-T6SS. We addressed H2- and H3-T6SS control by using transposon (Tn) mutagenesis and reporter fusions. We demonstrate that RsmA acts on most known T6SS genes, which has been undervalued in previous studies, and show that AmrZ is another global regulator of the T6SS. We also observe assembly of different T6SSs within a single cell, suggesting they are not mutually exclusive.

Results

Global and RsmA-Dependent Control of *P. aeruginosa* T6SSs. To identify regulators of H2-T6SS expression, we constructed a *lacZ* transcriptional fusion (A2tc) using the first gene in the H2-T6SS cluster (*tssA2*) (*SI Appendix*, Fig. S1), and inserted this reporter into the chromosome of *P. aeruginosa* PA14. Tn mutagenesis (16) was performed, and more than 85,000 Tn mutants were obtained. Those with altered β-galactosidase activity were isolated, and the position of the Tn insertion was mapped in 22 mutants (*SI Appendix*, Fig. S2 and Table S1). A Tn insertion in *rsmA* resulted in elevated levels of H2-T6SS expression (two- to threefold). Because RsmA is a posttranscriptional regulator, we constructed a *tssA2::lacZ* translational fusion (A2tl). The reporter was placed on the PA14 chromosome, and the *rsmA* gene was subsequently deleted. An increase of ∼36-fold in LacZ activity was observed in the *rsmA* background (Fig. 1*A*). RsmA was originally shown to repress H1-T6SS, which is confirmed here

Significance

Bacteria evolved molecular weapons to help them thrive in polymicrobial environments. The type VI secretion system (T6SS) is a gun loaded with a great diversity of bacterial toxins. On contact with neighboring cells, toxins are fired, and in the absence of immunity, the prey is killed, allowing the attacker to prevail. Each bacterium can be equipped with several distinct T6SSs, and it is unclear whether they are simultaneously active or whether each has a specific role in a particular environment. Here we showed that production of the three *Pseudomonas aeruginosa* T6SSs is orchestrated by global regulators. We suggest it may be possible for simultaneous assembly of multiple T6SSs within a single cell, priming it to fight a wide variety of organisms.

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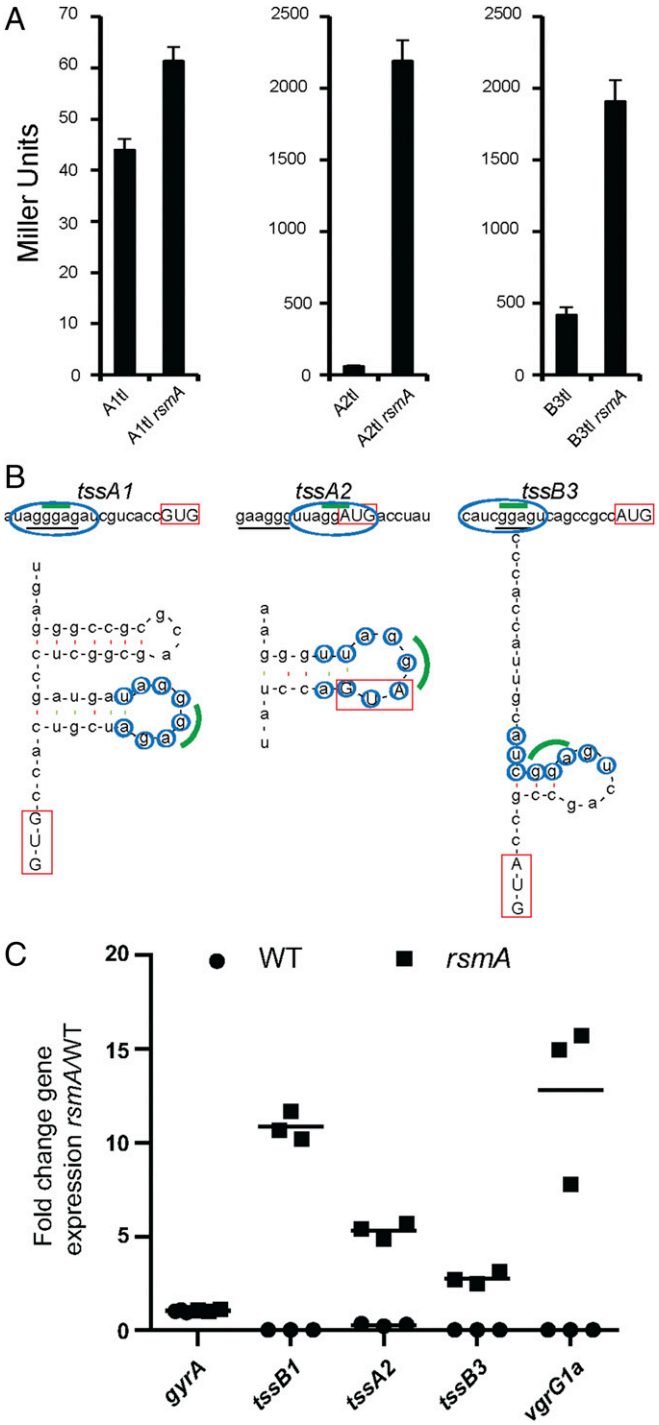

Fig. 1. RsmA controls all three T6SSs negatively. (*A*) β-galactosidase assay of PA14 and *rsmA* mutant (where indicated) carrying the H1-, H2-, or H3-T6SS translational fusion (A1tl, A2tl, or B3tl, respectively). Graphs represent mean ± SEM of five independent replicates (paired *t* test, $P < 0.005$, $P < 0.005$, and $P < 0.05$, respectively). (*B*) Predicted RsmA binding sites using Mfold for *tssA1*, *tssA2*, and *tssB3*. Blue ovals/circles, putative RsmA binding sites; green line, core GGA motif; red box, start codon; black underline, predicted RBS. (*C*) qRT-PCR analysis in PA14 or *rsmA* mutant for *tssB1* and *vgrG1a* (H1-T6SS), *tssA2* (H2-T6SS), *tssB3* (H3-T6SS), and the *gyrA* housekeeping gene control. Scatter plot of fold change with mean ($n = 3$). Statistical analysis was performed on the ΔΔCT values (ANOVA Bonferroni posttest $P > 0.05$, $P < 0.005$, $P < 0.005$, $P < 0.005$, and $P < 0.005$, respectively).

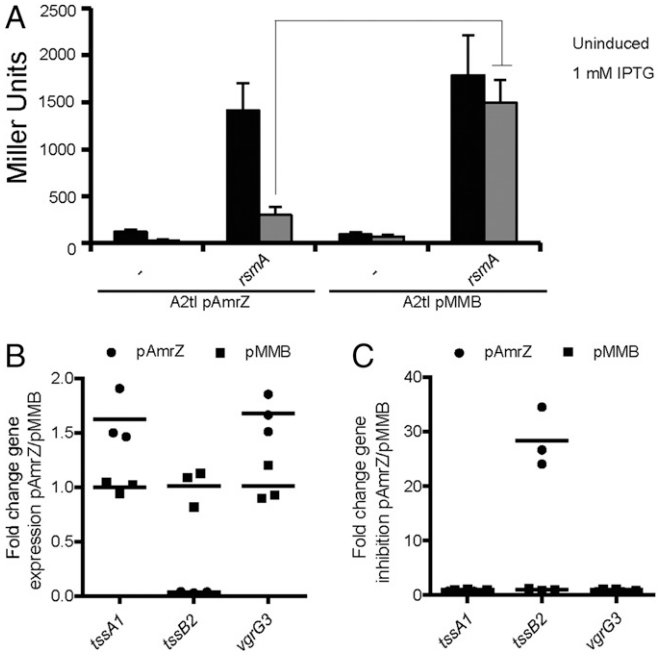

Fig. 2. AmrZ inversely regulates H2- and H1-/H3-T6SSs. (*A*) AmrZ is a negative regulator for H2-T6SS. Level of LacZ activity of A2tl after overexpression of *amrZ* (pAmrZ) or in the presence of vector (pMMB) ± induction with IPTG. Graph represents mean ± SEM; $n = 4$; ANOVA Bonferroni posttest $P < 0.001$. (*B*) AmrZ represses H2-T6SS (*tssB2*), but activates H1-T6SS (*tssA1*) and H3-T6SS (*vgrG3*). qRT-PCR was performed on PA14*rsmA* overexpressing *amrZ* (pAmrZ) and compared with vector control (pMMB). Scatter plot of fold change gene expression with mean ($n = 3$). Statistical analysis was performed on the ΔΔCT values (ANOVA Bonferroni posttest $P < 0.01$ for all genes). (*C*) Fold gene repression of data shown in *B*.

in PA14, using a *tssA1::lacZ* translational fusion (A1tl), although derepression is only about 1.4-fold (Fig. 1*A*). We then assessed the effect of RsmA on H3-T6SS by constructing a *tssB3::lacZ* translational fusion (B3tl), which displays a four- to fivefold increase in activity in the *rsmA* background (Fig. 1*A*). Previous analyses have shown that RsmA binds the *tssA1* mRNA in a region overlapping the ribosome binding site (RBS), which is thought to form a stem–loop structure (11) (Fig. 1*B*). Here, we identified putative RsmA binding sites on *tssA2* and *tssB3* mRNAs that both contain the core GGA motif (Fig. 1*B*), and Mfold analysis predicts these regions form a stem–loop structure (Fig. 1*B*) (17). Deletion of *rsmA* is likely to affect the stability of target RNA, as previously observed with H1-T6SS transcripts (11). We thus performed quantitative (q)RT-PCR analysis on *tssB1*, *tssA2*, *tssB3*, and *vgrG1a* genes (Fig. 1*C*), and all were up-regulated in a *rsmA* background from twofold (*tssB3*) to 12-fold (*vgrG1a*).

AmrZ Is a Global T6SS Transcriptional Regulator. Three distinct Tn insertions in and around the *amrZ* gene were selected that up-regulate (blue colony, B) or down-regulate (white colony, W) *tssA2* gene expression (*SI Appendix*, Fig. S2 and Table S1). Whereas two mutants (B1, B2) with Tn insertions close to the 5′ end of *amrZ* exhibited increased β-galactosidase activity, a third Tn insertion (W32), 297 bp upstream of the *amrZ* gene start codon, had decreased activity (*SI Appendix*, Figs. S2 and S3). We hypothesized that in B1/B2, the Tn insertion interrupts the gene or prevents transcription, whereas in W32, an outward reading promoter in the Tn induces *amrZ* expression. These results suggest that modulating AmrZ levels affects expression of H2-T6SS.

To probe this hypothesis, we engineered a deletion mutant of *amrZ* and a pMMB67HE-derivative overexpressing *amrZ* (pAmrZ). No significant difference in LacZ activity [*tssA2::lacZ* fusion (A2tc)] could be observed when comparing the wild-type

and *amrZ* mutant when grown in liquid culture (*SI Appendix*, Fig. S3*A*). However, β-galactosidase assays using bacteria scrapped from plates yielded a twofold increase in LacZ activity in the *amrZ* mutant, comparable to activity of the original B1 mutant (*SI Appendix*, Fig. S3*A*). Conversely, AmrZ overexpression (pAmrZ) resulted in a 6- to 11-fold reduction in LacZ activity (*SI Appendix*, Fig. S3*B*). Furthermore, in a *rsmA* mutant, the level of LacZ activity from a *tssA2::lacZ* translational fusion (A2tl) is high (~1,500 Miller units), but a fivefold reduction is seen on AmrZ overproduction (Fig. 2*A*), confirming AmrZ acts negatively on H2-T6SS. We conclude that two negative regulators act independently on H2-T6SS expression: AmrZ at the transcriptional and RsmA at the posttranscriptional level.

We then analyzed the effect of AmrZ on H1- and H3-T6SS expression by performing qRT-PCR on the PA14*rsmA* strain overexpressing *amrZ*. We confirmed a significant repression of *tssB2* (Fig. 2 *B* and *C*) and *tssA2* (*SI Appendix*, Fig. S3 *C* and *D*) and observed a significant induction of *tssA1* (H1-T6SS) and several genes encoding H3-T6SS components: *vgrG3*, *tssB3*, *hcp3*, and *tssA3* (Fig. 2 *B* and *C* and *SI Appendix*, Fig. S3 *C* and *D*). We conclude that AmrZ acts independent of RsmA, repressing H2-T6SS and activating expression of H1-T6SS and H3-T6SS.

AmrZ Binds Directly to T6SS Promoters. A consensus binding motif for AmrZ has been characterized in *P. aeruginosa* (18). We identified several degenerative versions of this motif in the upstream regions of *tssA1*, *tssA2*, and *tssB3* (*SI Appendix*, Fig. S1 and Table S2) and performed electrophoresis mobility shift assays. A His-tagged AmrZ protein was purified, and conditions were optimized by using DNA fragments previously shown to be bound or not by AmrZ (18, 19). Binding could be observed on the *tssA1* and *tssA2* upstream regions (Fig. 3*A*), whereas a weaker band shift occurs for the *tssB3* region, which is clear with 40–60 nM AmrZ (Fig. 3*B*). Smaller subfragments were used to demonstrate that only one of the putative binding sites for each upstream region was being bound by AmrZ (*SI Appendix*, Figs. S1 and S4 and Table S2) and confirmed that AmrZ binds to all three assessed T6SS promoter regions.

RsmA Controls Production of Hcp Proteins Negatively. We analyzed whether control on gene expression is reflected in protein production. Western blot analysis using specific antibodies confirmed production of Hcp1 (H1-T6SS) and Hcp2 (H2-T6SS) in a *rsmA* mutant (Fig. 4, *Upper*). To probe Hcp3 production, we engineered a chimeric *hcp3* gene on the PA14 chromosome, which encodes a V5-tagged version of Hcp3 (Hcp3V5). Hcp3V5 production was not detectable at 37 °C (Fig. 4, *Upper*), but was readily detected in a *rsmA* mutant when grown at 25 °C (Fig. 4, *Bottom*). Hcp1 and Hcp2 are also expressed in PA14 at 25 °C, with a modest increase in expression observed in a *rsmA* mutant (Fig. 4, *Bottom*).

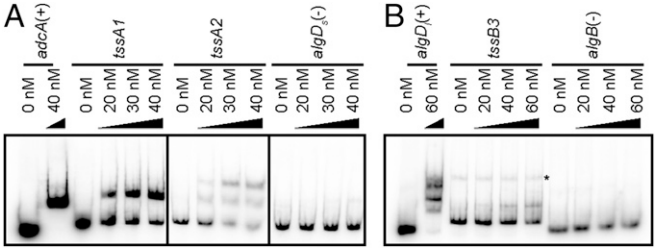

Fig. 3. AmrZ binds the promoter regions of T6SS genes. Each reaction contains 5 nM ^{32}P-labeled DNA and increasing concentrations of purified AmrZ, as indicated. Electrophoretic mobility shift assay was performed using DNA probes for (*A*) *tssA1*, *tssA2*, and (*B*) *tssB3*. In all cases, positive (*adcA*/*algD$_l$*) or negative (*algD$_s$*/*algB*) controls were used as previously published (18, 19). Asterisk indicates unspecific band.

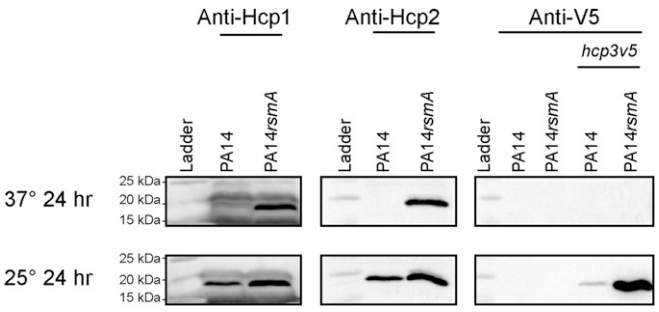

Fig. 4. RsmA controls all three Hcp proteins negatively. Western blot analysis comparing production of Hcp1, Hcp2, and Hcp3V5 at 37 °C (*Upper*) or 25 °C (*Lower*) in PA14 and *rsmA* mutant. Exposure times for Hcp blots are Hcp1, 600 s; Hcp2, 30 s; and Hcp3, 240 s.

H2- and H3-T6SS–Dependent Secretion Is Active in a *rsmA* Mutant. Hcp secretion is the hallmark of a functional T6SS. We assessed Hcp2 and Hcp3V5 secretion and observed that both are found in the supernatant fraction of a *rsmA* mutant, but were faintly detectable or absent in an H2- or H3-T6SS mutant (*SI Appendix*, Fig. S5 *A* and *B*). The H2-T6SS–dependent secretion of Hcp2 is very clear in both PA14 (Fig. 5*A* and *SI Appendix*, Fig. S5 *A* and *B*) and PAO1 (Fig. 6*A*), although low levels of Hcp2 in the supernatant of an H2-T6SS mutant were observed. Deletion of the H1- and H3-T6SS clusters (deleting both *hcp1* and *hcp3*) does not diminish the level of protein detected with the anti-Hcp2 antibody, suggesting it is not a cross-reacting protein (*SI Appendix*, Fig. S5*C*).

The identity of genuine T6SS effectors for H2- and H3-T6SS is poorly documented, but a few candidates have been described, such as PldA in the case of H2-T6SS (20, 21). PldA is encoded remotely from the H2-T6SS cluster and within the orphan *vgrG4b* cluster (PA3486-PA3488) (*SI Appendix*, Fig. S1). We engineered a chimeric gene encoding a PldA-Bla fusion and monitored its production using Western blot and a TEM β-lactamase antibody. PldA expression is increased in a *rsmA* background, which suggests RsmA negatively controls not only the expression of T6SS structural components but also the expression of effectors genes scattered on the chromosome (Figs. 6 and 7 and *SI Appendix*, Fig. S6*A*). We show that PldA is secreted in an H2-T6SS–dependent manner both in PAO1 or PA14 (Fig. 6*A* and *SI Appendix*, Fig. S6*B*) and, remarkably, in a VgrG4b-dependent manner (Fig. 6*B*), which suggests a direct connection with the VgrG4b spike and further validates the "à la carte delivery" concept that we previously proposed (6, 8).

Using qRT-PCR analysis, we confirmed that genes in most of the remote *vgrG* islands (*SI Appendix*, Fig. S1), including *vgrG2a*, *vgrG2b*, *vgrG4b*, *vgrG5*, and *vgrG6*, are induced in a *rsmA* background from fivefold (*vgrG2a*) to about 20-fold (*vgrG4b*) (Fig. 7*A*, *Left*). The genes encoding the VgrG-associated effectors [*tle4(tplE)*, *tle3*, *pldA*, *pldB*, and *PA14_69520*] were up-regulated in the *rsmA* mutant, ranging from twofold (*PA14_69520*) to 12-fold (*pldA*) (Fig. 7*A*, *Right*). Up-regulation coincides with protein production, as Western blot analysis using antibodies against VgrGs (VgrG2a, VgrG2b, and VgrG4b) or effectors such as PldB (PldB-Bla) showed clear de-repression in the *rsmA* mutant (Fig. 7*B*). Overall, our data demonstrate that relief of RsmA repression coordinates T6SS machinery assembly and effector delivery.

RsmA and AmrZ Repress H2-T6SS Bacterial Killing. We assessed the phenotypic effect of H2- and H3-T6SS, using a bacterial killing assay and *Escherichia coli* as the prey (6). The PA14 killing induced in a *rsmA* background was independent of H1-T6SS (Fig. 8*A* and *SI Appendix*, Fig. S7*A*), which was previously shown to be an antibacterial weapon in PAK or PAO1 (7, 22). The effect of H3-T6SS is also minor, whereas interruption of the *tssB2* gene in the H2-T6SS cluster abrogated killing. We have demonstrated that AmrZ negatively regulates H2-T6SS, and here show that overexpression of *amrZ* indeed alleviates H2-T6SS killing (Fig. 8*B*). We conclude that H2-T6SS is a major antibacterial weapon in PA14.

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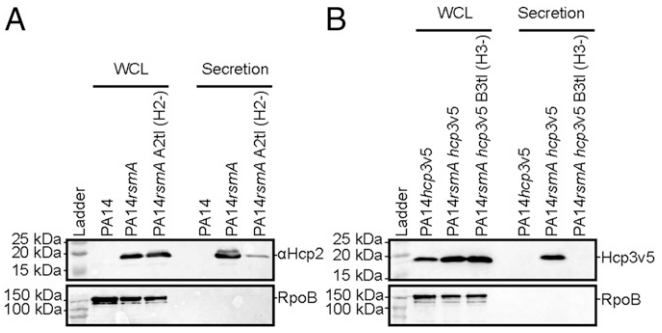

Fig. 5. H2-T6SS and H3-T6SS are functional in a *rsmA* mutant. (*A*) Hcp2 and (*B*) Hcp3 are used as readouts for T6SS-dependent secretion or presence in whole-cell lysate (WCL). Western blot analysis using (*A*) anti-Hcp2 on PA14, *rsmA* mutant, or *rsmA* H2-T6SS mutant (H2-) or (*B*) anti-V5 epitope to detect the tagged version of Hcp3 (Hcp3V5) in PA14, *rsmA* mutant, or *rsmA* H3-T6SS mutant (H3-). RNA polymerase (RpoB) is used as a lysis control.

Possible Assembly of Multiple T6SS Within a Single Cell. Given that all three T6SSs are coregulated, we used fluorescence microscopy to determine whether these systems could be coassembled within one cell. Fluorescent proteins were fused to the C terminus of the sheath component TssB encoded from each of the H1-, H2-, and H3-T6SS clusters and the corresponding recombinant plasmids introduced in PA14*rsmA*. Each TssB fusion allowed the viewing of extended sheath assemblies (Fig. 9*A*). The relative number of assembled H1-, H2-, and H3-T6SS machines was determined by quantifying the fluorescent foci (TssB1-Venus, TssB2-CFP, or TssB3-CFP) per total number of cells analyzed (*SI Appendix*, Fig. S8). The amount of TssB2-CFP foci was more than 10 times that observed for either TssB1-Venus or TssB3-CFP (*SI Appendix*, Fig. S8). To ensure that the visualized foci were the result of an assembled T6SS machine, each fluorescent fusion was expressed in a strain lacking all three T6SSs. No foci were observed for TssB1-Venus or TssB3-CFP out of a total of 44,036 and 32,000 cells analyzed, respectively; for TssB2-CFP, five foci were observed out of a total of 68,208 cells, and for TssB3-sfGFP, two foci were observed out of a total of 31,666 cells. We also confirmed that using the TssB2-CFP chimera does not affect T6SS function, as H2-T6SS–dependent killing remains fully effective (*SI Appendix*, Fig. S7*B*).

We then assessed whether different T6SSs may be simultaneously assembled in a single cell by introducing, pairwise, the various plasmids into PA14*rsmA*: TssB1-Venus with TssB2-CFP (Fig. 9*B*, *Upper*), TssB1-Venus with TssB3-CFP (Fig. 9*B*, *Middle*), and TssB2-CFP with TssB3-sfGFP (Fig. 9*B*, *Lower*). For any combination tested, both T6SS foci types may be found within the same cell at the same time (Fig. 9*B*), either at very distinct positions in the cell (e.g., H1- and H2-T6SS or H2- and H3-T6SS; Fig. 9*B*, *Upper* and *Lower*, respectively) or in close proximity (e.g., H1- and H3-T6SS; Fig. 9*B*, *Middle*).

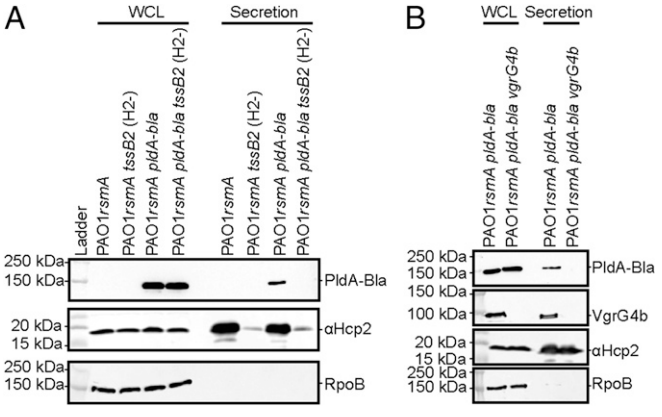

Fig. 6. Deletion of *rsmA* enables PldA secretion. Western blot analysis using anti-TEM to detect PldA-Bla expression shows expression in a PAO1*rsmA* mutant at 25 °C and secretion in a (*A*) H2-T6SS–dependent manner [*tssB2* mutant (H2-)] and (*B*) VgrG4b-depenedent manner (*vgrG4b* mutant). Anti-RNA polymerase (RpoB) is used as a lysis control and anti-Hcp2 as a control for H2-T6SS activity.

Discussion

The T6SS has a broad range of cellular targets and uses an armory of toxins and effectors to subvert or kill prey cells (1, 2). A bacterial species may carry several T6SSs; for example, three in *P. aeruginosa* (5), four in *Yersinia pseudotuberculosis* (23), and six in *Burkholderia pseudomallei* (24). In laboratory conditions, the T6SS is usually not expressed, suggesting environmental factors or host colonization, such as *P. aeruginosa* in the lungs of patients with cystic fibrosis (9), trigger T6SS assembly.

We demonstrate that in *P. aeruginosa*, the translational repressor RsmA negatively controls all T6SS clusters (H1-, H2-, and H3-T6SS) in PA14. This finding suggests H2- and H3-T6SS regulation by the RetS/Gac/Rsm cascade was undervalued and is not the privilege of H1-T6SS (25). We show that the RsmA-dependent control extends to orphan T6SS genes, such as those located in *vgrG* islands not associated with core T6SS clusters (6, 21). We concluded that RsmA is a central regulator imposing a tight and coordinated control that prevents T6SS-related messenger RNAs from being translated under yet-to-be-defined conditions.

We also identified AmrZ as a global transcriptional regulator of *P. aeruginosa* T6SSs. AmrZ acts as repressor or activator on a wide range of gene targets involved in *P. aeruginosa* virulence (26). Here, we showed that AmrZ positively influences both the H1- and H3-T6SS while having a negative control on the H2-T6SS. This is in agreement with available ChIP-Seq and RNA-Seq data investigating AmrZ (18). We also observed clear binding of AmrZ in the promoter region of these T6SS gene clusters. We concluded that AmrZ is a global regulator of T6SS genes, which can selectively promote or repress the transcription of a subset of T6SSs, whereas RsmA represses translation of all T6SS transcripts.

Regulatory events leading to RsmA and AmrZ expression are likely instrumental in fine-tuning expression of each individual T6SS. RsmA is downstream from a branched network in which two-component regulatory systems (25), phospho-relay (27) and c-di-GMP signaling (28), define the level of small RNAs (29), which sequester RsmA and alleviate T6SS repression. AmrZ is controlled by additional regulators such as the environmental stress sigma factor AlgU (also known as AlgT) (30). In our screen, a Tn hit in proximity to *algU* was identified (B100), as well as additional genes in the *alg* regulatory network, including

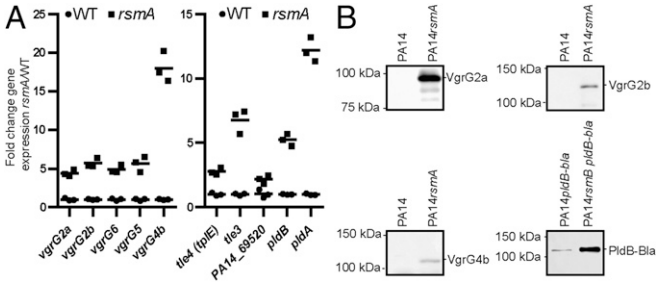

Fig. 7. RsmA controls remote *vgrG* and T6SS effector genes. (*A*) qRT-PCR on a subset of *vgrG* genes (*Left*) and genes encoding known/putative effectors (*Right*) (*SI Appendix*, Fig. S1) in PA14 (WT) or a *rsmA* mutant. Scatter plot of fold change with mean ($n = 3$). Statistical analysis was performed on the ΔΔCT values (ANOVA Bonferroni posttest, $P < 0.005$ for all genes). (*B*) Western blot analysis using specific antibodies against selected VgrG proteins (VgrG2a, VgrG2b, and VgrG4b) and the T6SS effector PldB (PldB-Bla fusion detected with anti-TEM).

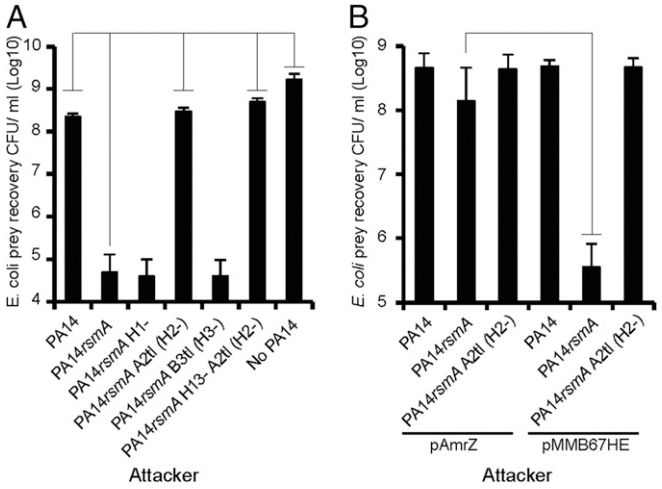

Fig. 8. RsmA and AmrZ repress H2-T6SS–dependent bacterial killing. (*A*) Quantification of bacterial killing assay after coincubation of *E. coli* and various PA14 attackers, including H1-T6SS, H2-T6SS, and H3-T6SS mutants, as indicated by H1-, H2-, and H3-, respectively. (*B*) H2-T6SS–dependent bacterial killing is significantly reduced after overexpression of *amrZ* (pAmrZ) compared with PA14*rsmA* mutant carrying the empty vector (pMMB67HE). Quantification is made using colony counts. Graphs represent mean ± SEM; $n = 3$; statistical significance is indicated ANOVA Dunnett's posttest $P < 0.05$.

algW, *algC*, and *mucP* (B26, W38b, and B39/B51; *SI Appendix*, Fig. S1 and Table S1). Interestingly, it was proposed that the two-component regulatory system AlgZ/AlgR manipulates the RsmAYZ pathway in *P. aeruginosa* (31), whereas the Gac/Rsm pathway represses the translation of AlgU (32).

The signals triggering the T6SS regulatory pathways can be many-fold. It has previously been proposed that quorum sensing and iron concentration influence H2- and H3-T6SS expression (14, 15, 33). Our study identified several genes associated with quorum sensing; for example, *lasR* and *rhlR* (B101 and W45) or *pqsA* (W43), which is required for the production of the 4-hydroxy-2-alkylquinoline signal (34) (*SI Appendix*, Fig. S1 and Table S1). We did not identify iron-related genes, but AmrZ represses many genes involved with iron procurement in Pseudomonads (18, 35). Other genes identified in our screen may have an indirect effect resulting from their role in central metabolism (*pncA*/B5, *dut*/W38b) and protein quality control (*lon*, W37a; *SI Appendix*, Fig. S1 and Table S1). We also pointed at significant differences in T6SS expression that depend on growth temperature. Whereas H1- and H2-T6SS are expressed at 37 °C, H3-T6SS is mainly produced at a lower temperature (e.g., 25 °C; Fig. 4, *Lower*). Furthermore, although induction of H1- and H2-T6SS is clear at 37 °C, at 25 °C, T6SS components are detectable even in a wild-type PA14 strain, indicating that expression no longer relies on deletion of *rsmA* (Fig. 4, *Lower*). An effect of temperature on T6SS expression was previously reported in *P. aeruginosa* (36), and one can suggest that the temperature response may be transmitted through the alternative stress sigma factor AlgU (37). In *Pseudomonas fluorescens*, it was also demonstrated that RetS contributes to the thermosensitivity of the Gac/Rsm-dependent gene expression (38). In *Y. pseudotuberculosis*, one of four T6SSs (T6SS4) displays higher level of expression at 26 °C and is under quorum sensing regulation (23).

In addition to the complexity of the regulatory network, it seems clear that expression of the different T6SSs depends on the *P. aeruginosa* isolate. Differences have been highlighted between the strains PAO1, PAK, and PA14, such as PA14 lacking the LadS sensor in the Gac/Rsm pathway (39). We observed differences in the expression of various T6SSs, as deletion of *rsmA* or *retS* in PA14 resulted in detectable levels of Hcp2 or TssB2 when grown at 37 °C, whereas similar deletion in PAK or PAO1 did not (*SI Appendix*, Fig. S9*A*). However, the H2-T6SS system in PAO1 is expressed and functional at 25 °C. In PA14, the role of H1-T6SS in a *rsmA* background is less crucial than H2-T6SS (Fig. 8*A* and *SI Appendix*, Fig. S7*A*), and despite Hcp1 being detectable in this background, TssB1 is not (*SI Appendix*, Fig. S9*B*). The potency of H2-T6SS in PA14 can also be suggested from the number of H2-T6SS sheaths visualized compared with H1- or H3-T6SS sheaths (*SI Appendix*, Fig. S8). It is also worth mentioning that in PA14, full expression of H1-T6SS involves the second RsmA-like encoding gene, *rsmF* (also called *rsmN*) (40). In the double mutant *rsmA*/*rsmF*, Hcp1 levels drastically increase, whereas TssB1 is now clearly detectable (*SI Appendix*, Fig. S9*B*). In contrast, no difference in H2-T6SS levels could be observed when comparing a *rsmA* or *rsmA*/*rsmF* mutant (*SI Appendix*, Fig. S9*B*). Overall, this supports the notion that different isolates of *P. aeruginosa* have defined networks to deploy their T6SS in different environments.

It is unclear how the different regulatory networks integrate to produce active T6SS machines (41), but in the environment or within a host, *P. aeruginosa* will have to face simultaneously the competition with other bacteria and resist predation from eukaryotic cells (e.g., macrophages or amoeba). Interestingly, RsmA has been suggested to be part of a bacterial danger-sensing circuit allowing *P. aeruginosa* to respond to kin cell lysis by enhancing the activity of H1-T6SS (13). Our data showing that RsmA represses not just H1-T6SS but all three T6SS support the idea that *P. aeruginosa*, on sensing kin cell lysis, relieves a RsmA posttranscriptional block of all three T6SS machines, enabling a coordinated response to attack. Our microscopy images suggesting that multiple systems may assemble within a single cell support such a defense/retaliation program in which RsmA acts to coordinate deployment of the

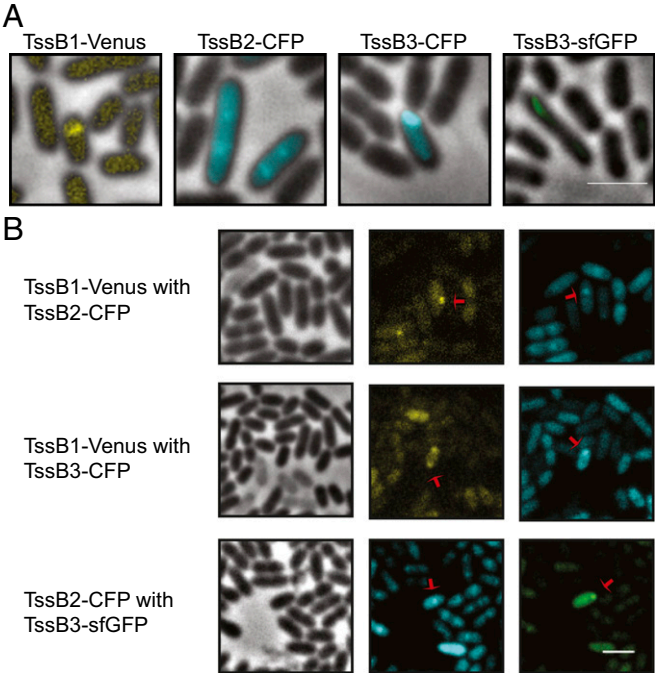

Fig. 9. Coassembly of multiple T6SS machines. (*A*) Assembly of extended T6SS sheaths as seen by fluorescence microscopy. Fluorescent fusions of TssB1-Venus, TssB2-CFP, TssB3-CFP, or TssB3-sfGFP were expressed in PA14*rsmA*. The images shown are representative of >100 fields analyzed from at least four independent experiments. (*B*) Fluorescent fusion combinations of TssB1-Venus with TssB2-CFP (*Upper*), TssB1-Venus with TssB3-CFP (*Middle*), or TssB2-CFP with TssB3-sfGFP (*Lower*) were coexpressed in PA14*rsmA*. (*Left*) Bright field channel. The arrows point to the foci of interest in cells that have two different T6SS machines assembled. The images shown are representative of ≥100 fields analyzed from at least two independent experiments. (Scale bars, 1 μm.)

complete arsenal of T6SS systems. In conclusion, the role of the T6SS and the complexity of the network controlling its assembly and functionality at all levels suggests it has evolved as a surveillance mechanism able to fight any organisms in any condition encountered.

Materials and Methods

Strains and plasmids are listed in *SI Appendix*, Table S3. Primers are listed in *SI Appendix*, Table S4. Gene deletions were constructed as previously described (42). Tn mutagenesis and identification of sites of integration was performed as previously outlined (16). RNA was isolated using TRIzol extraction and purified using the Qiagen RNeasy Mini kit (Qiagen). Real-time PCR was performed according to the manufacturer's protocol (Applied Biosystems or Sigma). Electrophoretic mobility shift assays were performed as previously described, using purified AmrZ (*SI Appendix*, Fig. S10) (18, 19). Assays for Western blotting, T6SS secretion, and T6SS killing were performed essentially as previously explained (6). Detailed information on methods and associated references are provided in *SI Appendix, Materials and Methods*.

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