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

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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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. S3A). However, β-galactosidase assays using bacteria scraped from plates yielded a twofold increase in LacZ activity in the amrZ mutant, comparable to activity of the original B1 mutant (SI Appendix, Fig. S3A). Conversely, AmrZ overexpression (pAmrZ) resulted in a 6- to 11-fold reduction in LacZ activity (SI Appendix, Fig. S3B). Furthermore, in a rsmA mutant, the level of LacZ activity from a tssB2::lacZ translational fusion (A2δ) is high (~1,500 Miller units), but a fivefold reduction is seen on AmrZ overproduction (Fig. 2A), 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. 3A), whereas a weaker band shift occurs for the tssB3 region, which is clear with 40-60 nM AmrZ (Fig. 3B). 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 32P-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) or negative (algD/algB) controls were used as previously published (18, 19). Asterisk indicates unspecific band.

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. SS A and B). The H2-T6SS-dependent secretion of Hcp2 is very clear in both PA14 (Fig. S4 and SI Appendix, Fig. SS A and B) and PA01 (Fig. S4), 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. S5C).

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. S6A). We show that PldA is secreted in a H2-T6SS-dependent manner both in PA01 or PA14 (Fig. S6A and SI Appendix, Fig. S6B) and, remarkably, in a VgrG4b-dependent manner (Fig. S6B), which suggests a direct connection with the VgrG4b spike and further validates the “a 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. 7A, Left). The genes encoding the VgrG-associated effectors [ile4(ile6), ile3, pldA, pldB, and PA14 69S20] were up-regulated in the rsmA mutant, ranging from twofold (PA14 69S20) to 12-fold (pldA) (Fig. 7A, 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. 7B). 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. 8A and SI Appendix, Fig. S7A), 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. 8B). We conclude that H2-T6SS is a major antibacterial weapon in PA14.
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 PA14ΔrsmA. Each TssB fusion allowed the viewing of extended sheath assemblies (Fig. 9A). 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 31,666 cells. We also confirmed for TssB1-Venus and TssB2-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. S7B).

We then assessed whether different T6SSs may be simultaneously assembled in a single cell by introducing, pairwise, the various plasmids into PA14ΔrsmA: TssB1-Venus with TssB2-CFP (Fig. 9B, Upper), TssB1-Venus with TssB3-CFP (Fig. 9B, Middle), and TssB2-CFP with TssB3-sfGFP (Fig. 9B, Lower). For any combination tested, both T6SS foci types may be found within the same cell at the same time (Fig. 9B), either at very distinct positions in the cell (e.g., H1- and H2-T6SS or H2- and H3-T6SS; Fig. 9B, Upper and Lower, respectively) or in close proximity (e.g., H1- and H3-T6SS; Fig. 9B, Middle).

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 vgrG and T6SS effector genes (Fig. 7A) was identified (B100), as well as additional genes in the alg regulatory network, including
PAO1 did not show differences in the expression of various T6SSs, as deletion of the LadS sensor in the Gac/Rsm pathway (39). We observed that RetS contributes to the thermosensitivity of the Gac/Rsm pathway in addition to the complexity of the regulatory network, it is unclear how the different regulatory networks integrate to produce active T6SS machines (41).

The signals triggering the T6SS regulatory pathways can be quorum sensing, which has an indirect effect resulting from their role in central metabolism (18, 35). Other genes identified in our screen may have an indirect effect resulting from their role in central metabolism (18, 35).

Quantification is made using colony counts. Graphs represent mean ± SEM; n = 3; statistical significance is indicated by ANOVA Dunnnett’s posttest P < 0.05.

**Fig. 8.** RsmA and AmrZ repress H2-T6SS–dependent bacterial killing. (A) Quantification of bacterial killing assay after coinoculation 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 PA14rsmA mutant carrying the empty vector (pMMB67HE). Quantification is made using colony counts. Graphs represent mean ± SEM; n = 3; statistical significance is indicated by ANOVA Dunnnett’s posttest P < 0.05.

**Fig. 9.** Coassembly of multiple T6SS machines. (A) Assembly of extended T6SS sheaths as seen by fluorescence microscopy. Fluorescent fusions of TssB1-Venus with TssB2-CFP (Upper), TssB1-Venus with TssB3-CFP (Middle), or TssB2-CFP with TssB3-sfGFP (Lower) were coexpressed in PA14ΔrsmA. 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 PA14ΔrsmA. (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 AimZ (SI Appendix, Fig. S10) (18, 19). Assays for Western blotting, T6SS secretion, and T6SS killing were performed essentially as previously explained (8). Detailed information on methods and associated references are provided in SI Appendix, Materials and Methods.

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Supplementary Information Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in SI Appendix, Table S3. *P. aeruginosa* strains were grown in or on tryptone soy broth (TSB), lysogeny broth (LB) or Vogel-Bonner medium (VBM) at either 25°C or 37°C with the addition of agar as required. *E. coli* strains were grown in or on LB, TSB, nutrient yeast broth NYB or terrific broth (TB) at 37°C with the addition of agar as required. Media was supplemented with antibiotics where appropriate, *P. aeruginosa*: streptomycin 2000 μg/ml, carbenicillin 50-300 μg/ml, irgasan 25 μg/ml, tetracycline 50-150 μg/ml and gentamycin 40-100 μg/ml or *E. coli*: streptomycin 50 μg/ml, ampicillin 50-100 μg/ml, tetracycline 15 μg/ml and kanamycin 50 μg/ml).

DNA manipulation

DNA isolation was performed using the PureLink Genomic DNA mini kit (Life Technologies). Isolation of plasmid DNA was carried out using the QIAprep spin miniprep kit (Qiagen). Primers used are shown in SI Appendix, Table S4 (Sigma). DNA fragments were amplified with either KOD Hot Start DNA Polymerase (Novagen) or standard Taq polymerase (NEB) as described by the manufacturer with the inclusion of Betaine (Sigma) or DMSO (Sigma). Restriction endonucleases were used according to the manufacturer’s specifications (Roche). DNA sequencing was performed by GATC Biotech. *P. aeruginosa* deletion mutants were constructed as described previously using 500 bp homologous regions and confirmed with external primers (SI Appendix, Table S4) (1). *amrZ* (PA14_20290) was amplified to construct a C-terminal V5 tag version (from pETDEST42 (Life Technologies)) and ligated into pMMB67HE. For protein purification *amrZ* was amplified and ligated into pET29a in frame with the sequence encoding a C-terminal His₆ tag. The DNA encoding the
C-terminus of VgrG4b (612-808 aa) was synthesised by Invitrogen GeneArt and subcloned into pET28a to construct pET28a-vgrG4b. For protein purification hcp3 was amplified, digested and ligated into pET28a in frame with a His6 tag. The coding regions of tssB1, tssB2 and tssB3 were amplified from Pseudomonas genomic DNA, cfp was amplified from pSEVA237C (de Lorenzo lab collection), ‘superfolder’ GFP (sfgfp) was amplified from mini-CTX-gfp (2) using KOD Hot Start DNA polymerase (Novagen). C-terminal fusions of each tssB gene with the respective fluorophore were generated by overlapping PCR (cfp and sfgfp) or by direct in-frame cloning (venus) in pME6032 (3).

**Transposon mutagenesis and screening**

Transposon mutagenesis was performed using pBT20 as outlined in (4). Briefly PA14tssB2tc::lacZ and SM10λpir pBT20 were grown overnight on LB plates, PA14 was then incubated at 43°C for 2 hr to inactivate the restriction system. Bacteria were collected, suspended, OD600 measured and cells were mixed 1:2, spot plated on LB plates to enable conjugation and incubated. Cells were collected, re-suspended and diluted before being spread plated onto LB agar containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Invitrogen), irgasan and gentamycin plates and incubated up to 4 days. Individual colonies were monitored for increased or decreased X-gal colour development twice daily. Transposon mutants of interest were isolated, grown, spot plated and confirmed via galactosidase assay essentially as previously described (5). Transposon insertion sites were mapped via arbitrary PCR (4).

**T6SS competition assays**

Competition assays were performed as per (6, 7) except that Top10 pRL662-gfp or DH5α pCR2.1 were used as the prey strains. Briefly overnight cultures were mixed 1:1 and spotted
on LB agar plates for 5 hr. LB agar contained 1 mM IPTG or 1% Arabinose if induction of pAmrZ or pJN105tssB2-cfp was required. Spots were recovered, suspended, serial diluted and spotted on to LB, LB X-gal, LB gentamycin and/or PIA plates respectively to enable colony counts as required.

**Secretion assays**

For secretion assays *P. aeruginosa* strains were grown overnight in TSB and subcultured to OD<sub>600</sub> 0.1 and grown at 37°C or 25°C for 6 or 24 hr, respectively (or as otherwise indicated) with agitation. Culture supernatants were prepared as previously described using trichloroacetic acid precipitation (8). An OD<sub>600</sub> equivalent of 0.1 or 1 of cell lysate and supernatant protein samples, respectively were loaded on to SDS-PAGE gels for analysis by western blotting.

**Protein Purification**

BL21 Star (λDE3) pAmrZ-His<sub>6</sub> cells were sub-cultured to an OD<sub>600</sub> 0.1 in one litre of NYB and grown at 37°C to an OD<sub>600</sub> 0.7 prior to induction with 1 mM IPTG for 5 hours. Cells were then harvested and frozen at -80°C. The frozen pellet was thawed on ice, suspended in 30 ml of buffer (20 mM Imidazole, 50 mM Tis, 500 mM NaCl) supplemented with cOmplete, EDTA-free protease inhibitor cocktail (Roche) and egg white lysozyme (Sigma) was added to 1 mg/ml and incubated for 45 min prior to probe sonication. The soluble fraction was separated by centrifugation at 18000g for 45 minutes at 4°C and then passed through a syringe driven 0.45 μm filter. Clarified extract was applied to Ni<sup>2+</sup>–NTA columns (Hi-Trap, GE Healthcare) using an ÄKTA Prime. The column was washed with 10-15 column volumes of buffer before switching over to the elution buffer (500 mM Imidazole, 50 mM Tis, 500 mM NaCl). The sample was assessed for purity using SDS-PAGE gels and coomassie
staining and the band corresponding to AmrZ-His$_6$ confirmed with western blot analysis (See **Fig. S10**). The sample was then diluted into buffer (50 mM Tis, 500 mM NaCl) and quantification was performed using a Pierce BCA protein assay kit following the manufactures instructions and using BSA as standards. *E. coli* B834 (DE3) carrying pET28a-vgrG4b, along with *E. coli* BL21 (λDE3) harbouring pET28a-hcp3 were grown in terrific broth, induced and harvested. The frozen pellet was suspended in buffer supplemented with cOmplete, EDTA-free protease inhibitor cocktail (Roche) prior to disruption by French press. Clarified extract was applied to Ni$^{2+}$–NTA columns (Hi-Trap, GE Healthcare) using an ÄKTA Prime for purification.

**Western blot analysis**

SDS-PAGE and western blotting were performed as described in (6). Briefly, proteins were resolved in 8% (VgrGs, RpoB), 12% (PldA, PldB, RpoB) or 15% (Hcps, TssBs and AmrZ) gels using the Mini-PROTEAN system (Bio-Rad) by electrophoresis and transferred to nitrocellulose membrane (GE Healthcare). Membranes were blocked in 5% milk (Sigma) in Tris-buffered saline pH 8 with 0.1% Tween-20 (TBST) prior to incubation with primary antibodies. Monoclonal antibodies were used at the following dilutions: anti-RNA polymerase (Biolegend) at 1:5000, anti-beta lactamase (Bla) (GeneTex International Corporation) at 1:1000 and anti-V5 (ThermoFisher) at 1:5000. Polyclonal primary antibodies described previously were used at a dilution of 1:1000. These include anti-Hcp1 (8), anti-Hcp2 (9), anti-TssB1 (10), anti-TssB2 (9), anti-VgrG2a and anti-VgrG2b (11). The polyclonal anti-VgrG4b and anti-Hcp3 antibodies were also used at 1:1000. Membranes were washed with TBST before incubation with HRP-conjugated secondary antibodies (Sigma) at a dilution of 1:5000. All monoclonal primary antibodies required anti-mouse secondary antibodies, whereas the polyclonal antibodies all required anti-rabbit secondary antibodies.
Signals were detected using the Novex ECL HRP Chemiluminescent substrate (ThermoFisher) or the Luminata Forte Western HRP substrate (Millipore) using a LAS-3000 Fuji Imager. ECL detection and a white light image of the ladder were taken on a Fuji LAS-3000, separately. Adobe Photoshop was used to adjust the brightness/contrast of the tiff images prior to overlaying and merging of the two layers using the multiply tool followed by merge layers.

**Antibody Production**

Custom Anti-VgrG4b antibodies were raised against the purified C-terminus of VgrG4b (612-808) with Eurogentec following their immunisation protocols. Antibodies against purified full-length Hcp3 protein was raised similarly.

**Electrophoretic mobility shift assays**

Electrophoretic mobility shift assays (EMSA) were performed as previously described (8, 9). Using the previous published controls (8, 9). Each reaction contained 40 mM NaCl, 4 mM Tris, 4 mM MgCl2 (pH 8.0), 4% glycerol (W/V), 100 mg/ml BSA (non-specific protein control), 150 ng/μl poly-deoxy-inosinic-deoxy-cytidylic acid (d[(I-C)]) (Sigma) (non-specific DNA control), 5 nM 32P-labeled DNA probe and purified AmrZ in the nM range. The 10 μl reactions were incubated at room temperature for 20 min prior to the addition of 2 μl of native loading dye and loading of the entire sample. Reactions were analysed on 4% (w/v) native polyacrylamide gels run at 200 V for 22 minutes prior to drying. Gels were visualised on a Typhoon FLA7000 Phosphorimager (GE Healthcare).

**Real Time PCR**
Overnight cultures were subcultured in TSB, grown to early exponential phase and harvested in RNAlater (Ambion). RNA was isolated using TRIZol extraction and purified using the Qiagen RNeasy Mini kit (Qiagen) followed by DNase digestion using PureLink DNase Set (Invitrogen). Purified RNA was stored at −80°C in nuclease-free water. cDNA was synthesised from 200 ng of RNA using SuperScript III Reverse Transcriptase (Invitrogen) and random hexamers (Applied Biosystems) according to the manufacturer’s protocol. Real time PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems) or KiCqStart SYBR Green qPCR ReadyMix (Sigma) on an Applied Biosystems StepOnePlus Real-Time PCR machine. The primers used for amplification are shown in SI Appendix, Table S4. Real-time analysis was performed on RNA from three independent cultures in duplicate and quantification of rpoD gene expression served as an internal control. The relative expression ratios were calculated using the delta-delta method relative to PA14 wild-type or PA14rsmA pMMB as indicated.

**Microscopy Procedure**

Strains for visualizing coexpression of multiple T6SS systems in situ were as follows: PA14rsmA, PA14rsmA H123 and PA14rsmA H13 A2tl (H2-) with combinations of fluorescent fusions pME6032tssB1-venus, pJN105tssB2-cfp, pJN105tssB3-cfp and/or pMMB67tssB3-sfgfp (SI Appendix, Table S3, S4). *P. aeruginosa* strains for microscopy were grown overnight in TSB with appropriate antibiotic(s) at 37°C, then diluted to an OD$_{600}$ = 0.1 in 10 ml fresh TSB plus appropriate antibiotic(s) and grown to OD$_{600}$ = 0.3 at 25°C. Plasmid expression was induced with IPTG (50 μg/ml) for strains containing pME6032 or pMMB67HE plasmids, or with arabinose (1%) for strains containing pJN105 plasmids. Cells growing exponentially (OD$_{600}$ = 0.8-1.0) were harvested by centrifugation (8000 g, 3 min) and resuspended in PBS to a final OD$_{600}$ of 10 units. One microliter of resuspended culture
was spotted on a glass-bottomed dish (Ibidi 35 mm μ-dish) and covered with a 1% PBS agarose pad. Cells were imaged using a Zeiss Axiovert 200 inverted microscope fitted with Hamamatsu Flash 4 (2048x2048 pixel) camera for fast low-light imaging, a pE4000 CoolLed Led Illumination source and a 100x 1.4 objective. A 500 nm LED was used to excite TssB1-Venus with an exposure time of 1000 ms using a YFP filter; a 435 nm LED was used to excite TssB2-CFP and TssB3-CFP with exposure times of 300 ms and 100 ms respectively using a CFP filter; and finally, a 500 nm LED was used to excite TssB3-sfGFP with an exposure time of 300 ms using a GFP filter. The LED and dichroic filter sets were selected based upon the spectral profiles for each fluorophore to ensure optimised excitation and emission settings were used for each of the relevant co-expressed fluorescent fusions.

**Image analysis**

Microscopy images were analysed in Fiji (10). The Bleach Correction plugin was used on time-lapse image series (15). To determine the number of cells across all fields to be analysed the background was subtracted from brightfield DIC images using a filtered Gaussian blur image with Sigma (radius) = 20 and the ‘Image Calculator’ Fiji tool. Huang segmentation was then used within the ‘Threshold’ tool to select all the cells in each field, with the tool ‘Analyse Particles’ finally used to quantify the number of cells in each field. The range used for ‘Analyse Particles’ was set at 30-infinity pixels, to ensure that small areas of background brightfield particles (i.e. not cells) were excluded. The number of cells counted by this automated method was initially manually checked on a subset of images to ensure the process was a true reflection of the number of cells present in all subsequent analyses. The number of cells containing foci was then counted manually by using the ‘Multi-Point’ tool in Fiji. At least 10 separate randomly selected fields of view were analysed for each fluorescent fusion
in PA14*rsmA* which contained between 185 276 and 319 440 total cells or in PA14*rsmA* H123 or PA14*rsmA* H1H3 A2tl (H2-) which contained between 31 666 and 68 208 total cells.

**Bioinformatics and statistical analysis**

DNA sequences were retrieved from the Pseudomonas Genome Database (16). Binding motifs were investigated using Mfold (17), FUZZNUC (EMBOSS) and BPROM. DNA and amino acid sequence searches were executed using SMART, InterPsoScan, Pfam and BLAST. Statistical analysis was performed using GraphPad Prism version 5 as indicated in the text.

**Additional references**


Figure S1
Figure S2
Figure S3
Figure S4
Figure S5
Figure S6
Figure S7
Figure S8
Figure S9
Figure S10
**Supplementary Figures Legends**

**Fig. S1.** Schematic representation of *P. aeruginosa*’s T6SS and vgrG clusters from PA14. Gene names or numbers are indicated below each gene cluster. PAO1 gene numbers are indicated above each cluster for the vgrG encoding genes for reference (NP = not present). Colored genes indicated T6SS structural components (blue), vgrGs (yellow), toxin/immunity pairs (orange/green), other known T6SS genes (dark grey) and not currently associated with the T6SS in light grey. Genes checked via qRT-PCR are indicated by an asterisk. Schematic of upstream regions used for AmrZ binding assays are show and hashed region indicates the sub-fragment bound by AmrZ.

**Fig. S2.** Expression of the H2-T6SS gene cluster in selected PA14 transposon mutants. (A) Level of β-galactosidase activity of the A2tc transcriptional fusion in selected mutants are shown. Mutants have been grouped as described in the key. (B) Location of transposon insertion in proximity to amrZ (PA14_20290). Insertion mutants B1 (14 bp upstream of gene) and B2 (26 bp into gene), which may prevent AmrZ expression (indicated by dotted cross), resulted in increased expression of A2tc. In contrast, insertion mutant W32 (298 bp upstream of amrZ) which may increase read through of amrZ (indicated by dotted arrow) resulted in reduced expression of A2tc.

**Fig. S3.** AmrZ inversely regulates the H2- and H3-T6SS. (A) AmrZ is a negative regulator of the H2-T6SS. Deletion of amrZ relieves repression of tssA2 when grown on agar plates. Beta-galactosidase assay was performed on PA14, PA14A2tc, PA14A2tc armZ and PA14A2tc B1 grown in either liquid LB or on LB plates. (B) Beta-galactosidase assay showing that overexpression of AmrZ represses transcription of tssA2 (A2tc) which is absent in the uninduced or vector only (pMMB67HE). Strains were grown in liquid culture. (C) AmrZ represses the H2-T6SS (tssA2) but activates the H3-T6SS (tssB3, hcp3 and tssA3). qRT-PCR was performed on PA14rsmA overexpressing amrZ (pAmrZ) and compared to vector control (pMMB). Scatter plot of fold change gene
expression with mean (N = 3). Statistical analysis was performed on the \( \Delta \Delta CT \) values (ANOVA Bonferroni post-test \( tssB1 \) \( P > 0.05 \), \( P < 0.05 \) for all other genes). (D) Fold gene repression of data shown in C.

**Fig. S4. Dissection of AmrZ binding sites.** EMSA was performed using sub fragments of the DNA probes used in figure 3; (A) \( tssA1_1, tssA1_2 \), (B) \( tssA2_1, tssA2_2 \) and (C) \( tssB3_1 tssB3_2 \). In all cases AmrZ only binds to one of the sub fragments from each upstream region. Each reaction contains 5 nM \(^{32}\)P-labelled DNA and increasing concentrations of purified AmrZ. In all cases, positive (\( adcA \)) or negative (\( algD_s \)) controls were used as previously published (18, 19). See also Fig. S1 for schematic of AmrZ binding fragments used in this study.

**Fig. S5.** The H2-T6SS is functional in a \( rsmA \) mutant and Hcp2 depends upon this system for secretion. (A) Hcp2 is only faintly detectable when the H2-T6SS is disrupted (PA14\(rsmA\) A2tl \( \text{(H2-)} \)) but is still secreted when either the H1-T6SS is deleted (PA14\(rsmA\) H1-) or the H3-T6SS is disrupted (PA14\(rsmA\) B3tl \( \text{(H3-)} \)) after 5 h of growth from an \( \text{OD}_{600} \) of 0.1 at 37 degrees. (B) Hcp2 is drastically reduced when the H2-T6SS is disrupted (PA14\(rsmA\) A2tl \( \text{(H2-)} \)) but is still secreted when either the H1-T6SS is deleted (PA14\(rsmA\) H1-) or the H3-T6SS is disrupted (PA14\(rsmA\) B3tl \( \text{(H3-)} \)) after 8 h of growth from an \( \text{OD}_{600} \) of 0.1 at 37 degrees. RNA polymerase (RpoB) is used as a lysis control. (C) Deletion of the H1-T6SS cluster including \( hcp1 \) and the H3-T6SS cluster including \( hcp3 \) does not diminish the level of protein detected with the anti-Hcp2 antibody.
Fig. S6. PldA and VgrG4b are expressed in a *rsmA* mutant. (A) Western blot analysis using anti-TEM to detect PldA-Bla expression, or anti-VgrG4b shows enhanced expression of both proteins in PAO1*rsmA* at 25°C. (B) Deletion of *rsmA* enables PldA secretion. Western blot analysis using anti-TEM to detect PldA-Bla expression shows expression in a PA14*rsmA* mutant at 25°C and secretion in a H2-T6SS-dependent manner (A2tl (H2-)). Anti-RNA polymerase (RpoB) is used as a lysis control and anti-Hcp2 as a control for H2-T6SS activity.

Fig. S7. *P. aeruginosa* H2-T6SS dependent killing of *E. coli*. (A) Serial dilution of the 1:1 bacterial mix including *E. coli* GFP+ and the PA14 attackers as listed. *E. coli* is recovered significantly less when co-incubated with PA14*rsmA* as compared to PA14 and the killing is specifically H2-T6SS dependent as it is not alleviated in either the H1- or H3-T6SS mutants. (B) H2-T6SS killing assays demonstrating that the H2-T6SS is as functional in PA14*rsmA* as PA14*rsmA* carrying either pJN105 or pJN105tssB2-cfp. *P. aeruginosa* strains were mixed individually with *E. coli* DH5α carrying pCR2.1 which expresses β-galactosidase. *Pseudomonas* strains were grown in the presence of arabinose 1% prior to the killing assay and arabinose 1% was included in the LB agar for the 5 h contact time to enable constant expression of TssB2-CFP. Recovered bacteria were diluted and spotted on X-gal plates. The level of blue color indicates survival of *E. coli* when the H2-T6SS is disabled (PA14*rsmA* A2tl).

Fig. S8. Significantly more H2-T6SS (TssB2-CFP) foci where observed then either H1-T6SS (TssB1-Venus) or H3-T6SS (TssB3-CFP) foci. Quantification of relative numbers of TssB1-Venus, TssB2-CFP and TssB3-CFP foci present in PA14*rsmA* after induction. Cells were grown at 25°C until the OD reached 0.3, at which point expression of the fluorescent fusions was induced by addition of IPTG or arabinose, with cells being harvested during mid-exponential phase and imaged directly. The mean number of foci per total PA14*rsmA* cells + SEM is represented based upon at least 40 separate fields
of view captured over two independent days. Total numbers of cells analysed were 284 985 for TssB1-Venus, 185 276 for TssB2-CFP and 319 440 for TssB3-CFP. Statistical analyses were performed using a one-way ANOVA with multiple comparisons (Tukey) comparing all 3 samples (P <0.005).

**Fig. S9.** Differential expression of T6SSs (A) Western blot analysis of regulator mutants showing higher levels of expression of H2-T6SS components in PA14 mutants compared to the same mutants in PAK or PAO1. Bacteria were grown at 37°C. (B) Western blot analysis showing that strong expression of the H1-T6SS components Hcp1 and TssB1 requires the double rsmArsmF mutant. H2-T6SS components have enhanced expression in an rsmA mutant but no additional expression was observed in the rsmArsmF mutant.

**Fig. S10.** Representative blot for the purification of AmrZ used in EMSAs. (A) Coomassie stained SDS-PAGE gel and (B) western blot using anti-His antibody to detect AmrZ-His$_6$. Fraction 12 and 13 were used for EMSAs.

**Table S1.** Location of Transposon insertion in mutants with altered transcription of tssA2tc

**Table S2.** Identification of putative AmrZ binding sites upstream of tssA1, tssA2 and tssB3

**Table S3.** Strain and Plasmid List
Table S4. Primer List
### Table S1: Location of Transposon insertion in mutants with altered transcription of tssA2tc

<table>
<thead>
<tr>
<th>Mutant code</th>
<th>Site of integration</th>
<th>Gene inserted into/near</th>
<th>PAO1</th>
<th>Gene name</th>
<th>Description</th>
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<td>1747943</td>
<td>PA14_20290/PA14_20300</td>
<td>PA3385/PA3384</td>
<td>amrZ(algZ)/phnC</td>
<td>Upstream of DNA binding protein (alginate and motility regulator Z), ABC phosphonate transporter ATP-binding protein</td>
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<td>B2</td>
<td>1747903</td>
<td>PA14_20290</td>
<td>PA3385</td>
<td>amrZ(algZ)</td>
<td>DNA binding protein (alginate and motility regulator Z)</td>
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<td>5789115</td>
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<td>PA4917/PA4918</td>
<td>pncA</td>
<td>NAD biosynthetic process/Nicotinamide</td>
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<td>B11</td>
<td>3920247</td>
<td>PA14_44030</td>
<td>PA1583</td>
<td>sdiA</td>
<td>Insertion in Succinate dehydrogenase (A subunit), effect likely on sdhB (B subunit)</td>
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<td>AlgW protein, Alginate pathway</td>
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<td>mucP</td>
<td>Putative membrane-associated zinc metalloprotease</td>
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<td>7316</td>
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<td>PA0905</td>
<td>rsmA</td>
<td>Regulator of secondary metabolism (carbon storage regulator)</td>
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<td>PA14_54430/PA14_54450</td>
<td>PA0762/PA0761</td>
<td>algU/nadB</td>
<td>Sigma factor AlgU/AlgT/o22 / L-aspartate oxidase</td>
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<td>PA3477</td>
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<td>W38b</td>
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Table S2: Identification of putative AmrZ binding sites upstream of tssA1, tssA2 and tssB3

<table>
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<tr>
<th>Gene</th>
<th>Predicted AmrZ site?</th>
<th>Bp from ideal</th>
<th>3’ end relative to ATG (bp)</th>
<th>Strand relative to gene</th>
<th>Location</th>
<th>Putative Site</th>
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<td>-ve</td>
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<tr>
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<td>AATTTTGCAAACC</td>
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Note: Fragment in red text is bound by AmrZ in SI appendix, Fig. S4.
Table S3: Strain and Plasmid List

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<tr>
<th>Strain/Plasmid</th>
<th>Relevant Characteristics</th>
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<td>Wild type</td>
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<tr>
<td>PAK</td>
<td>Wild type</td>
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<tr>
<td>PA14 ΔrsmA</td>
<td>PA14rsmA::lacZ (PA14_00990) deletion mutant</td>
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<tr>
<td>PA14ΔrsmA ΔpldA-bla</td>
<td>PA14ΔrsmA ΔpldA-bla fusion with βla Beta Lactamase</td>
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<tr>
<td>PA14 ΔrsmA ΔpldA-bla ΔtssA2</td>
<td>PA14ΔrsmA ΔpldA-bla ΔtssA2 fusion with βla Beta Lactamase</td>
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<td>PA14ΔrsmA ΔclpV3 deletion mutant</td>
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<td>PA01ΔrsmA ΔclpV3 deletion mutant</td>
</tr>
<tr>
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<td>PA01ΔrsmA ΔclpV3 ΔtssA1 fusion with βla Beta Lactamase</td>
</tr>
<tr>
<td>PA14ΔrsmA ΔclpV3 ΔtssA1</td>
<td>PA14ΔrsmA ΔclpV3 ΔtssA1 fusion with βla Beta Lactamase</td>
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<td>Blunt cloning vector, 2Kb/KmR</td>
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<td>pMBM87HE</td>
<td>pMBM87HE::lacZ (PA14_24302) deletion mutant</td>
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<td>pCR2.1</td>
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Plasmids

pCR-BluntII-TOPO | Blunt cloning vector, 2Kb/KmR |
| pMBM87HE | pMBM87HE::lacZ (PA14_24302) deletion mutant |
| pCR2.1 | pCR2.1 cloning vector |

References:

- Difco: F' recA1 galE44 thi-1 recA1 rpsL22 gyrA96 deoR mapR4 punB28 806lacZΔM15 lacY1-argF151 (U169, hsdR17) F' (λpir) recA1 rpsL22 gyrA96 deoR mapR4 thi-1 (λpir) F' (λpir) recA1 rpsL22 gyrA96 deoR mapR4 thi-1 (λpir) F' (λpir) recA1 rpsL22 gyrA96 deoR mapR4 thi-1 (λpir) F' (λpir) recA1 rpsL22 gyrA96 deoR mapR4 thi-1 (λpir)

- E. coli DH5α

- Host strain for pPHG101 replication; ΔlacI lacZΔM15 lacY1-argF151 (U169, hsdR17)

- SM12804

- Host strain for pPHG101 replication; ΔlacI lacZΔM15 lacY1-argF151 (U169, hsdR17)

- BL21 Star (DE3) | $\nabla$ ampβ lacZΔM15 lacY1-argF151 (U169, hsdR17)

- HB21 (DE3) | $\nabla$ ampβ lacZΔM15 lacY1-argF151 (U169, hsdR17)

- Host strain for pPHG101
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<tr>
<th>Primer</th>
<th>Name</th>
<th>Clifomodulator Sequence (5')</th>
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OAL3404  
OAL821  
OAL820  
OAL3023  
OAL3022  
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OAL511  
OAL1030  
OAL3032  
OAL3039  
OAL2137  
OAL2136  
OAL3030  
OAL3043  
OAL3042  
OAL536  
OAL2135  
OAL2134  
OAL4025  
OAL4022  
OAL3091  
OAL2989  

5’sfGFP  
tssB3_sfGFP_F  
3’	tssB3  
3’	tssB2  
5’	tssB1  
vgrG3	R  
tssB2	R  
pldA(tle5a)	R  
pldB(tle5b)	R  
PA14_69520	R  
tle3	F  
tle4(tplE)	F  
vgrG4b	R  
vgrG2b	R  
vgrG2b	F  
vgrG2a	R  
tssB3amrZ200R  
tssA1amrZ93F  
algB69  
algB68  

GCATCGGAGTCAGCCGCC  
GCAGCAGCAGGAGGAGGAATGGTGAGCAAGGGCGA  
GGGGTACCCGCGCCTGCGGCTCGTCGTC  
TCCCCATGTCGTTGATCATG  
AGGCCGTGAGCAGGGATAC  
CTTCAACGGCAGTTCGATTT  
GATAGACGCCGTCCCAGTA  
CTGCTGCAGAGCAACGTC  
TTGGTGAAGTTCAGCGACAG  
GATCATGCCCTTCATCGTCT  
GCAGCTCCTTCTTCTCGATG  
GCCTTCAACGTACCGAACGT  
AAGAGGTTGGACCCCTTGAT  
CCTCCACGAAAAGGAACTCA  
TCTACTACCGGCGCAAGTTC  
GTCCAGGGTAAGGAGCACAA  
AGCCATCGCCCAGAAGAT  
GGAGCCGGGAAAGACGTT  
TGCCGCAAGGCTTGTCAA  
CGCATGCCAGCCTTTCTGA  
CCTCTGGTCCGTCACTGGCGAC  
CCCTCTTTGGGAATTTTCCTATTC  

3’	primer	sfGFP  
Generation	of	C-term	CFP	fusion	with	linker	using	pSEVA237C	as	template  
To	amplify
tssB3	(PA2365)	with	a	linker
to
generate	overlapping	product	with	CFP	linker  
To	amplify
tssB2	(PA1657)	with
to
generate	overlapping	product	with	CFP	linker  
Cloning
tssB1	into	pME6032-Cterm-venus  
qRT-PCR	rpoD	(housekeeping
gene)  
qRT-PCR	tssA1	(PA0082/PA14_00990)	100	bp  
qRT-PCR
tssA1	(PA0082/PA14_00990)	100	bp  
qRT-PCR
tssA3	(PA14_34140/PA2360)  
qRT-PCR	tssA2	PA14_43050/PA1656)  
qRT-PCR
tssB1	(PA14_01010/PA0083)  
qRT-PCR	pldA(tle5a)	(PA14_18970/PA3487)  
qRT-PCR	pldB	(PA14_67220/PA5089)  
qRT-PCR	tle3	(PA14_03200/PA0260)  
qRT-PCR	tle4	(PA14_44910/PA1510)  
qRT-PCR	tle4	(PA14_44910/PA1510)  
qRT-PCR	vgrG4b	(PA14_18985/PA3486)  
qRT-PCR	vgrG6	(PA14_69550)  
qRT-PCR	vgrG6	(PA14_69550)  
qRT-PCR	vgrG2b	(PA14_03220/PA0262)  
Use	with	OAL2986	to	amplify	a	94	bp	fragment	with	2	amrZ	binding	site  
algD  
algB	negative
to
test	AmrZ	binding
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Mini-prep: planarik
pET15b(l)

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