Supplemental Experimental Procedures

Quantitative time-lapse microscopy

Single colonies of *P. aeruginosa* Δ*retS* Δ*sspB* pPSV38::*sspB* and *P. aeruginosa* Δ*retS* Δ*sspB tsi6*-D4 pPSV38::*sspB* were resuspended in LB and 1-2 μL of each suspension was spotted onto a 1.5% (*w*/*v*) agarose growth pad containing 100 μM IPTG (prepared using Vogel Bonner minimal media containing 0.2% (*w*/*v*) sodium nitrate and 0.01% (*w*/*v*) casamino acids) and sealed.

Microscopy data were acquired using NIS Elements (Nikon) acquisition software on a Nikon Ti-E inverted microscope with a 60× oil objective, automated focusing (Perfect Focus System, Nikon), a xenon light source (Sutter Instruments), and a CCD camera (Clara series, Andor). Cell division times were calculated from time-lapse sequences acquired at 5 min intervals. At least three fields were acquired for each experimental group and analyzed as described previously (LeRoux et al., 2015).

Tsi6 depletion assays

Tsi6 depletion assays were performed using an inducible protein degradation system optimized for use in *P. aeruginosa* (Castang and Dove, 2012). Briefly, a chromosomally encoded C-terminal degradation tag, DAS+4, was introduced at the native *tsi6* locus in *P. aeruginosa* strains lacking the *sspB* gene. SspB recognizes proteins containing the DAS+4 tag, and delivers them to the ClpXP protease for degradation. To allow for controllable degradation of Tsi6-DAS+4 (Tsi6-D4), a plasmid with IPTG-inducible *sspB* expression was used. Strains harboring this plasmid were patched on LB agar in the presence or absence of 100 μM IPTG. Images of plates were acquired using a Pentax WG-3 digital camera.

Protein expression and purification

To prevent cytotoxicity during overexpression in *E. coli*, all Tse6 constructs were co-expressed with Tsi6. For the $Tse6_{222\text{-}CT}$, $Tse6_{252\text{-}CT}$, $Tse6_{265\text{-}CT}$, and $Tse6_{282\text{-}CT}$ truncations, the appropriate *tse6* gene fragment was cloned into MCS-1 of pETDuet-1 (Novagen) using the BamH1/HindIII restriction sites. The *tsi6* gene was cloned into

MCS-2 using the NdeI/XhoI restriction sites. The resulting plasmids encode N-terminally His_6-tagged Tse6 truncations and untagged Tsi6. Full-length Tse6 was cloned into MCS-1 of pETDuet-1 using the NcoI/HindIII restriction sites and a 3' primer that fuses a His_8-tag to the C-terminus of the protein. Genes encoding Tsi6, EF-Tu^{PA}, and EF-Tu^{EC} without stop codons were cloned into pET29b (Novagen) resulting in C-terminal His_6-tagged fusions. The *eagT6* and *vgrG1* genes were cloned into MCS-1 and MCS-2 of pRSFDuet-1 using the NcoI/HindIII and NdeI/XhoI restriction sites, respectively.

For protein expression, the above plasmids were transformed into *E. coli* BL21 pLysS cells (Novagen). For expression of the Tse6 complex for EM analysis, pETDuet-1::*tse6*-his_8 ::*tsi6* and pRSFDuet-1::*vgrG1* ::*eagT6* were co-transformed into *E. coli* BL21 pLysS cells. For all expression constructs, stationary phase overnight cultures were used to inoculate 4L of 2 x YT broth and the cultures were grown to mid-log phase in a shaking incubator at 37°C. Upon reaching an OD_{600} of approximately 0.6, protein expression was induced by the addition of 1 mM IPTG followed by further incubation at 37°C for 4 h. Cells were harvested by centrifugation at 6000 *g* for 15 minutes, followed by resuspension in 50 mL of buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole). Resuspended cells were then ruptured by sonication (6 pulses, 30 seconds each) and cellular debris was removed by centrifugation at 25,000 *g* for 35 minutes. Cleared cell lysates were then purified by nickel affinity chromatography using a 1 mL HisTrap™ FF Ni-NTA cartridge connected to an AKTA FPLC purification system (GE Healthcare). Using an automated purification program, proteins were loaded onto the column and unbound proteins were removed using 50 mL of buffer A. Bound proteins were then eluted in using a linear imidazole gradient to a final concentration of 300 mM. The purity of each protein sample was assessed by SDS-PAGE followed by Coomassie Brilliant Blue staining. All protein samples were dialyzed into 20 mM Tris-HCl, 150 mM NaCl.

Cells expressing selenomethionine-incorporated $Tse6_{282\text{-}CT}$–Tsi6 and Tsi6 I37M were grown in SelenoMethionine Medium Complete (Molecular Dimensions) using the expression conditions described above. Cell lysis and nickel affinity purification were also performed as described above except that all buffers contained 1 mM tris(2-carboxyethyl)phosphine (TCEP).

The buffers used for the purification of the detergent-bound Tse6-Tsi6-VgrG1-EF-Tu^{EC} complex were identical to those described above with the addition of 0.2% β-D-decylmaltopyranoside. A second purification step using a Superose 6 Increase 5/150 column was performed in order to exchange the complex into a detergent with a lower critical micelle concentration prior to electron microscopy analysis (20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.03% β-D-dodecylmaltopyranoside).

For experiments requiring free Tse6, a denaturation and refolding strategy was employed to separate the various Tse6 truncations from Tsi6 and EF-Tu^{EC}. First, proteins were buffer exchanged into denaturation buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 8 M urea) by spin filtration. Next, denatured proteins were run over a Ni-NTA column to remove Tsi6 and EF-Tu^{EC}. On-column refolding was achieved by washing the column with renaturation buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl) followed by elution of refolded protein using 20 mM Tris-HCl pH 8.0, 300 mM NaCl, 300 mM imidazole. Protein samples were subsequently dialyzed into 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% (*v*/*v*) glycerol.

Crystallization and data collection

For crystallization of selenomethionine-incorporated Tsi6, Ni-NTA purified protein was concentrated to 10mg/mL by spin filtration (10 kDa cutoff, Millipore) and screened against commercially available crystallization screens (Microlytic). Diffraction quality crystals appeared after 7 days in a solution containing 0.1 M imidazole:HCl pH 8.0, 2.5 M NaCl. Crystals were cryoprotected in crystallization buffer supplemented with 25% (*v*/*v*) glycerol and X-ray diffraction data were collected using beamline X29A at the National Synchrotron Light Source (NSLS). Low-resolution (90% attenuator, 90 images, 2° Δφ oscillation, 0.4s exposure) and high-resolution (No attenuator, 360 images, 1.0° Δφ oscillation, 0.5s exposure) datasets were collected on an ADSC Q315r CCD detector with a 250 mm crystal-to-detector distance. Data were merged, integrated and scaled using HKL2000 (Table S1).

For crystallization of selenomethionine-incorporated Tse6$_{282\text{-}CT}$–Tsi6, Ni-NTA purified complex was concentrated to 25mg/mL by spin filtration and screened for crystallization conditions. Diffraction quality crystals appeared after 3 days in a condition

containing 20% (*w*/*v*) PEG 3350, 0.2M ammonium iodide. Crystals were cryoprotected in crystallization buffer supplemented with 20% (*v*/*v*) ethylene glycol and X-ray diffraction data were collected using beamline 8.2.2 at the Advanced Light Source (ALS). A single dataset (360 images, 1.0° Δφ oscillation, 1.0s exposure) was collected on an ADSC Q315r CCD detector with a 200 mm crystal-to-detector distance. Data were merged, integrated and scaled using HKL2000 (Table S1).

For crystallization of the $Tse6_{265\text{-}CT}$–$EF\text{-}Tu^{PA}$ complex, refolded $Tse6_{265\text{-}CT}$ was mixed with Ni-NTA purified $EF\text{-}Tu^{PA}$ in a 1:1 molar ratio and concentrated to 12mg/mL by spin filtration. The concentrated complex was then screened for crystallization conditions, and small microcrystals were observed in 20% (*w*/*v*) PEG 3350, 0.2M ammonium formate pH 6.6. Optimization of this condition led to growth of diffraction quality crystals in 22% (*w*/*v*) PEG 3350, 0.2M ammonium formate pH 6.6. Crystals were cryoprotected in crystallization buffer supplemented with 20% (*v*/*v*) ethylene glycol and X-ray diffraction data were collected using beamline X29A at the National Synchrotron Light Source (NSLS). Low-resolution (95% attenuator, 90 images, 2° Δφ oscillation, 0.4s exposure) and high-resolution (No attenuator, 360 images, 1.0° Δφ oscillation, 0.4s exposure) datasets were collected on an ADSC Q315r CCD detector with a 400 mm crystal-to-detector distance. Data were merged, integrated and scaled using HKL2000 (Table S1).

Structure determination and refinement

The structures of Tsi6 and $Tse6_{282\text{-}CT}$–Tsi6 were solved by Se-SAD using the AutoSol wizard in the Phenix GUI (Adams et al., 2010). For both structures, the density modified electron density maps were of excellent quality and allowed for automated model building using the AutoBuild wizard in the Phenix GUI. For Tsi6, the electron density allowed for complete building of the model whereas for $Tse6_{282\text{-}CT}$–Tsi6 a near-complete model of the complex was built, with the exception of an internal segment of $Tse6_{282\text{-}CT}$ spanning residues 400-408. Minor model adjustments were made manually in COOT between iterative rounds of refinement, which was carried out using Phenix.refine. The progress of the refinement was monitored by the reduction of R_{work} and R_{free} (Table S1).

The structure of $Tse6_{265\text{-}CT}$–$EF\text{-}Tu^{PA}$ was solved by molecular replacement using the Phaser-MR GUI in Phenix. The GDP-bound form of $EF\text{-}Tu^{EC}$ (PDB code 1EFC) was used as a search model and upon structure solution additional electron density corresponding to $Tse6_{265\text{-}CT}$ was clearly visible. $Tse6_{265\text{-}CT}$ was built manually in COOT using the high-resolution $Tse6_{282\text{-}CT}$ structure as a guide. The asymmetric unit contains two molecules of $EF\text{-}Tu^{PA}$ and two molecules of $Tse6_{265\text{-}CT}$. Non-crystallographic symmetry (NCS) and secondary structure restrains were used during the course of refinement. The electron density for domains two and three of $EF\text{-}Tu^{PA}$ molecule B was significantly worse than for molecule A. However, refinement after removal of these domains led to a significant (2-3%) increase in R_{work} and R_{free}. Therefore, these domains were left built with their relative positions restrained to that of the equivalent domains in molecule A.

Isothermal titration calorimetry

All protein and ligand solutions were degassed prior to experimentation. ITC measurements were performed with a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA). Titrations consisted of 25-30 10-μL injections with 180-s intervals between each injection. The ITC data were analyzed using the Origin software package (version 5.0, MicroCal, Inc.) and fit using a single-site binding model.

Fluorescence-based $NAD(P)^+$ glycohydrolase assay

All $NAD(P)^+$ glycohydrolase assays were performed in 96-well plate format. Refolded Tse6 constructs at the concentrations indicated were incubated with 0.7 mM $NAD(P)^+$ in a final volume of 100 μL. Reactions were carried out at 37°C and the incubation time was adjusted for each enzyme concentration to ensure <10% of the total substrate was consumed. Parallel reactions containing 1.5 molar equivalents of Tsi6 were setup for each enzyme concentration. Reactions were terminated by the addition of 50 μL of 6M NaOH and incubated in the dark at room temperature for 30 min to allow for development of the base-catalyzed fluorescent breakdown product of $NAD(P)^+$. Samples were analyzed using a Synergy H1 plate reader (Biotek) at excitation and emission wavelengths of 360 nm and 420 nm, respectively. The amount of $NAD(P)^+$ hydrolyzed in

each reaction was determined by interpolation from a $NAD(P)^+$ standard curve.

Determination of relative $NAD(P)^+$ levels from cell lysates

E. coli strains harboring expression plasmids for $Tse6_{282\text{-}CT}$ or $Tse6_{282\text{-}CT}$ and Tsi6 were grown in LB media at 37°C to mid-log phase prior to induction of protein expression with 0.1% (*w*/*v*) rhamnose ($Tse6_{282\text{-}CT}$) and 0.1 mM IPTG (Tsi6). 1h post-induction, cultures were diluted to OD_{600} = 0.5 and 500 µL of cells were harvested by microcentrifugation. Cells were lysed in 0.2 M NaOH, 1% (*w*/*v*) cetyl trimethylammonium bromide (CTAB) followed by treatment with 0.4 M HCl at 60°C for 15 min. After neutralization with 0.5 M Tris base, samples were then mixed with an equal volume of NAD/NADH-Glo™ Detection Reagent or NADP/NADPH-Glo™ Detection Reagent (Promega). Luciferin bioluminescence was measured continuously using a Synergy H1 plate reader. The slope of the luciferin signal from the linear range of the assay was used to determine relative $NAD(P)^+$ concentrations between samples.

For $NAD(P)^+$ quantitation in *P. aeruginosa* monocultures, approximately 100 µg of a parental strain (Δ*retS* Δ*sspB* pPSV38::*sspB*) and each indicated Tsi6 depletion strain were collected from LB agar plates and resuspended in LB media containing 0.1 M IPTG. After shaking for 1h at 37°C, cultures were diluted to OD_{600} = 0.5 and 500µL of cells were harvested by microcentrifugation. Cell lysis and relative $NAD(P)^+$ concentrations were determined as described above.

Quantification of NAD^+ levels in a Tse6-sensitive recipient strain during interbacterial competition was performed by first measuring CFUs and [NAD^+] of a control competition (Δ*retS* vs. Δ*retS*). This NAD^+/CFU ratio was then used to determine the amount of NAD^+ contributed by donor cells and the amount of NAD^+ expected in non-intoxicated recipient cells in a Δ*retS* vs. Δ*retS* Δ*tse6* Δ*tsi6* competition. The Tse6-dependent reduction in NAD^+ reported for Δ*tse6* Δ*tsi6* recipient cells is the difference between the measured [NAD^+] of the intoxicated strain and the expected [NAD^+] for non-intoxicated recipient cells.

Immunoprecipitation assays

Log phase *P. aeruginosa* cultures were spun down and resuspended in lysis buffer containing 20mM Tris-HCl pH 7.5, 150mM NaCl, 2% glycerol and 1% Triton X-100. After rupture by sonication, cleared cell lysates were incubated with anti-VSV-G agarose beads for 1 h at 4°C. Beads were then washed four times in wash buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 2% glycerol and 0.1% Triton X-100) before bound proteins were eluted by boiling in Laemmli sample buffer.

Identification of Tse6 interacting partners by mass spectrometry

Immunoprecipitated proteins were analyzed by SDS-PAGE and visualized using the SilverQuest™ staining kit (Invitrogen). Bands of interest with then excised and prepped for MS analysis as per the manufacturer's instructions. Samples were then vacuum dried and resolved in 10 µL of ACN/H_2O/FA (5/95/0.1, *v*/*v*/*v*) for LC-MS/MS analysis. The samples were analyzed in duplicate using a nanoLC-MS system comprising a nanoflow LC (NanoAcquity; Waters Corporation, Milford, MA) and a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive™, Thermo Fisher, San Jose, CA). For each run, 4 µL of sample was loaded on a 100 µm i.d. x 20 mm 200Å, 5 µm C18AQ in-house packed pre-column at flow rate of 4 µL/min for 5 min, using a loading buffer of ACN/H_2O/FA (5/95/0.1, *v*/*v*/*v*). Peptides were separated on a 75 µm i.d. x 180 mm 100 Å, 5 µm C18AQ analytical column in a 95-min gradient at a flow rate of 250 nL/min, using mobile phase A of 0.1% formic acid in water and mobile phase B of 0.1% FA in acetonitrile. The gradient elution started at 5% mobile phase B, increased to 35% at 60 min, 80% at 65 min and held at 80% in 5 min before a 25 min re-equilibration at 5%.

Mass spectrometry data were collected in positive electrospray ionization mode using a data dependent acquisition method, employing one full MS scan for m/z range 350-2000 at 70K resolution and consecutive MS/MS scans for top 20 abundant ions at 17.5 K resolution within a dynamic exclusion of 30 seconds. Precursor ions selected from the MS scan were isolated with an isolation width of 2 *m*/*z* for higher energy collisional dissociation (HCD) at energy NCE = 35.

MS data were analyzed by MaxQuant (version 1.5.0.25) using standard settings and a UniprotKB database of *Pseudomonas aeruginosa* PAO1 (Cox and Mann, 2008). Peptide-spectrum matches (PSM) and protein identifications were filtered at a false

discovery rate of 0.01. Label-free quantification, MS/MS spectral counts, were extracted and used for statistical analysis of differential expression by using QSpec tool (version 1.2.2) (Choi et al., 2008).

Identification of the products generated by Tse6-catalyzed breakdown of NAD^+

1μM $Tse6_{282\text{-}CT}$ or 1μM $Tse6_{282\text{-}CT}$–Tsi6 complex was incubated with 1mM NAD^+ for 15 minutes at room temperature. Proteins were then removed from the samples by spin filtration (3 kDa cutoff, Millipore) and the reaction products were analyzed in a LC-MS system comprising a Waters Acquity UPLC system and a Waters Synapt – G1 QTOF tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA). For each run, 2 μL of sample was injected onto a Hypersil Gold 2.1x100 reverse phase C18 column at flow rate of 0.3 mL/min, using a mobile phase consisting of ACN/H_2O (2/98, *v*/*v*). Reaction products were eluted using an 8.0mL linear gradient to 100% acetonitrile.

For NAD^+ and nicotinamide, mass spectrometry data were collected in positive electrospray ionization mode whereas negative ionization mode was used for ADP-ribose. Data were acquired in centroid mode spanning an *m/z* range of 100-1000. The resulting mass spectra were analyzed using the MassLynx 4.1 MS software package.

Membrane fractionation

Total cell membranes of *P. aeruginosa* strains were isolated by centrifugation of cleared cell lysates at 50,000 g for 30 minutes. Membranes were washed twice with wash buffer (20mM Tris-HCl pH 7.5, 150mM NaCl) before being solubilized by the addition of Laemmli sample buffer.

Secretion assays

Overnight cultures of *P. aeruginosa* strains were used to inoculate 2 ml of LB at a ratio of 1:500. Cultures were grown at 37°C with shaking to mid-log phase, and cell and supernatant fractions were prepared as described previously (Hood et al., 2010).

Western blotting

Western blot analyses of protein samples were performed using rabbit α-VSV-G (diluted 1:2500, Sigma), rabbit α-Tse1 (diluted 1:2000), mouse α-OprF (diluted 1:10000) or mouse α-RNAP (diluted 1:5000, Sigma) and detected with α-rabbit or α-mouse horseradish peroxidase-conjugated secondary antibodies (diluted 1:5000, Sigma). Western blots were developed using chemiluminescent substrate (SuperSignal West Pico Substrate, Thermo Scientific) and imaged with a FluoroChemQ (ProteinSimple).

Electron Microscopy and Image Analysis

4 µL of each sample was adsorbed for 2 min at 25 ˚C onto glow-discharged carbon-coated copper grids. The grids were washed twice with the appropriate purification buffer and negatively stained with 0.75 % uranyl formate.

Samples were imaged with a JEOL1400 microscope equipped with a LaB_6 cathode operated at 120 kV. Images were recorded at a nominal magnification of 50,000x on a 4k x 4k CCD camera F416 (TVIPS) using minimal dose conditions. Particles were manually selected, aligned, and classified using reference free alignment and ISAC classification procedures implemented in SPARX and EMAN2 (Hohn et al., 2007) (Tang et al., 2007). The Tse6–Tsi6–VgrG1–EF-Tu^{EC} and the Tse6–Tsi6–VgrG1–EagT6–EF-Tu^{EC} data sets contained 10,702 and 11,844 particles, respectively. For the 3D reconstructions, the best ISAC classes were chosen and 3D models were calculated using sxviper of the SPARX software package. Using the set of raw particle images, the reconstruction was then further refined by iterative projection matching implemented in SPARX until convergence was achieved. The resolution of the final reconstructions, estimated using a Fourier shell correlation criterion of 0.5, were calculated to be 21.5 Å for the Tse6-Tsi6-VgrG1-EagT6-EF-Tu^{EC} complex and 19.4 Å for Tse6-Tsi6-VgrG1-EF-Tu^{EC} complex. Local resolution calculations for the Tse6-Tsi6-VgrG1-EF-Tu^{EC} complex were performed as described previously using a 20 Å sphere for windowed FSC calculations at 0.5 (Anger et al., 2013). Crystal structures were fitted with the 'Fit in map' tool as rigid bodies into the final density using Chimera software. Cross correlation coefficients for Tse6-Tsi6-VgrG1-EagT6-EF-Tu^{EC} and Tse6-Tsi6-VgrG1-EF-Tu^{EC} of 0.74 and 0.77 were calculated using the 'Fit in map' tool in Chimera (Pettersen et al.,

2004). The accession numbers for the EM density maps of the detergent-free and detergent-bound complexes are EMD-3113 and EMD-3112, respectively.

To visualize the position of the C-terminal His_8-tag of Tse6, the complex was incubated for 60 min on ice with 5 nM Ni-NTA Nanogold solution (Nanoprobes) at a ratio of 9:1 (*v*/*v*) and subsequently applied to a normal glow-discharged carbon-coated copper grid and washed/stained as described above.

Software

Protein sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). The Tse6 sequence alignment was prepared using the HMMER web server (http://hmmer.janelia.org/) (Finn et al., 2011). All structure figures were generated using Chimera (Pettersen et al., 2004).

Supplemental Tables

Table S1. X-ray data collection and refinement statistics. Related to Experimental Procedures.

	$Tse6_{282\text{-}CT}$–Tsi6	Tsi6	$Tse6_{265\text{-}CT}$–$EF\text{-}Tu^{PA}$
Data Collection			
Wavelength (Å)	0.979	0.979	1.075
Space group	$P6_5$	$C222_1$	$P4_12_12$
Cell dimensions			
a, b, c (Å)	83.2, 83.2, 83.8	54.5, 101.0, 93.1	176.1, 176.1, 86.3
α, β, γ (°)	90.0, 90.0, 120.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	50.00 – 1.37 (1.37 – 1.42)[a]	47.98 – 1.95 (2.02 – 1.95)	50.00 – 3.50 (3.63 – 3.50)
Unique reflections	69121	19003	17642
R_{merge} (%)[b]	8.2 (61.7)	9.7 (68.7)	11.1 (74.2)
$I/\sigma I$	33.5 (1.8)	26.0 (4.6)	39.4 (5.4)
Completeness (%)	99.9 (99.5)	99.8 (100.0)	100.0 (100.0)
Redundancy	15.2 (9.4)	15.0 (14.4)	27.6 (27.2)
Refinement			
R_{work} / R_{free} (%)[c]	14.6/16.4	19.2/23.6	23.5/27.8
Average B-factors (Å^2)	17.9	47.6	120.2
No. atoms			
Protein	3672	1455	8082
Ligands	0	0	54
Water	366	98	0
Rms deviations			
Bond lengths (Å)	0.022	0.007	0.003
Bond angles (°)	1.713	0.933	0.784
Ramachandran plot (%)[d]			
Total favored	98.7	98.9	95.6
Total allowed	100.0	100.0	99.5
Coordinate error (Å)[e]	0.11	0.22	0.50
PDB code	4ZV0	4ZUY	4ZV4

[a]Values in parentheses correspond to the highest resolution shell.

[b]$R_{merge} = \Sigma \Sigma |I(k) - \langle I \rangle| / \Sigma I(k)$ where $I(k)$ and $\langle I \rangle$ represent the diffraction intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements.

[c]$R_{work} = \Sigma ||F_{obs}| - k|F_{calc}|| / |F_{obs}|$ where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. R_{free} is the sum extended over a subset of reflections excluded from all stages of the refinement.

[d]As calculated using MOLPROBITY (Chen et al., 2010).

[e]Maximum-Likelihood Based Coordinate Error, as determined by PHENIX (Adams et al., 2010).

Table S2. Strains used in this study. Related to Experimental Procedures.

Organism	Genotype	Reference
P. aeruginosa PAO1	ΔPA4856	(Goodman et al., 2004)
	ΔPA4856 attB::*lacZ*, Tet^R	(Whitney et al., 2014)
	ΔPA4427 ΔPA4856	(Silverman et al., 2013)
	ΔPA4427 ΔPA4856 *tsi6*-D4	This study
	ΔPA0077 ΔPA4427 ΔPA4856	This study
	ΔPA0077 ΔPA4427 ΔPA4856 $tse6^{L270E}$	This study
	ΔPA0077 ΔPA4427 ΔPA4856 *tsi6*-D4	This study
	ΔPA0077 ΔPA4427 ΔPA4856 $tse6^{L270E}$ *tsi6*-D4	This study
	ΔPA4856 *tse6*-V	This study
	ΔPA4856 $tse6^{L270E}$-V	This study
	ΔPA4856 $tse6^{D396A}$-V	This study
	ΔPA0077 ΔPA4856 *tse6*-V	This study
	ΔPA0077 ΔPA4856 $tse6^{L270E}$-V	This study
	ΔPA0090 ΔPA4856 *tse6*-V	This study
	ΔPA0090 ΔPA4856 $tse6^{L270E}$-V	This study
	ΔPA0091 ΔPA4856 *tse6*-V	This study
	ΔPA0094 ΔPA4856 *tse6*-V	This study
	ΔPA0093 ΔPA4856	(Whitney et al., 2014)
	ΔPA0092 ΔPA0093 ΔPA4856 attB::*lacZ*, Tet^R	(Whitney et al., 2014)
	ΔPA2774 ΔPA2775 ΔPA4856 attB::*lacZ*, Tet^R	(Whitney et al., 2014)
E. coli SM10 λpir	*thi thr leu tonA lac Y supE recA*::RP4-2-Tc::Mu	
E. coli DH5a	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 D(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Novagen
E. coli BL21 (DE3) pLysS	F^- ompT gal dcm lon $hsdS_B(r_B^-$ $m_B^-)$ λ(DE3) pLysS(cm^R)	Novagen

Table S3. Plasmids used in this study. Related to Experimental Procedures.

Plasmid	Relevant features	Reference
pEXG2	Allelic replacement vector containing *sacB*, Gm^R	(Rietsch et al., 2005)
pSW196	MiniCTX1 plasmid, Tet^R	(Baynham et al., 2006)
pPSV35-CV	Expression vector with *lacI*, *lacUV5* promoter, C-terminal VSV-G tag, Gm^R	(Mougous et al., 2006)
pPSV38-CV	Expression vector with *lacI*, *lacUV5* promoter, C-terminal VSV-G tag, Gm^R	(Castang et al., 2008)
pSCrhaB2-CV	Expression vector with *PrhaB*, Tmp^R	(Cardona and Valvano, 2005)
pETDuet-1	Co-expression vector with *lacI*, T7 promoter, N-terminal His_6 tag in MCS-1, Amp^R	Novagen
pRSFDuet-1	Co-expression vector with *lacI*, T7 promoter, N-terminal His_6 tag in MCS-1, Kan^R	Novagen
pET29b	Expression vector with *lacI*, T7 promoter, C-terminal His_6 tag, Kan^R	Novagen
pEXG2::ΔPA0077	*tssM1* deletion allele in pEXG2	(Mougous et al., 2006)
pEXG2::ΔPA0090	*clpV1* deletion allele in pEXG2	(Mougous et al., 2006)
pEXG2::ΔPA0091	*vgrG1* deletion allele in pEXG2	(Hood et al., 2010)
pEXG2::PA0092_DAS+4	For generating strains encoding Tsi6 with a C-terminal DAS+4 tag (DENYSENYADAS)	This study
pEXG2::PA0093-VSV-G	For generating strains encoding Tse6 with a C-terminal VSV-G tag	This study
pEXG2::PA0093_L270E	For generating strains encoding Tse6 L270E point mutant	This study
pEXG2::PA0093_D396A	For generating strains encoding Tse6 D396A point mutant	This study
pEXG2::ΔPA0092 ΔPA0093	*tse6-tsi6* deletion allele in pEXG2	(Whitney et al., 2014)
pEXG2::ΔPA4427	*sspB* deletion allele in pEXG2	(Silverman et al., 2013)
pEXG2::ΔPA4856	*retS* deletion allele in pEXG2	(Goodman et al., 2004)
pSW196::*lacZ*	*lacZ* in miniCTX1 plasmid	(Vance et al., 2005)
pPSV35-CV::PA0094	Complementation vector for *eagT6*	This study
pPSV38-CV::PA4427	Expression vector for *sspB*	(Castang and Dove, 2012)
pSCrhaB2-CV:: PA0093_282-430	Expression vector for $tse6_{282\text{-}430}$	This study
pETDuet-1::PA0093_222-430 ::PA0092	Co-expression vector for $tse6_{222\text{-}430}$ and *tsi6*	This study
pETDuet-1::PA0093_252-430 ::PA0092	Co-expression vector for $tse6_{252\text{-}430}$ and *tsi6*	This study
pETDuet-1::PA0093_265-430 ::PA0092	Co-expression vector for $tse6_{265\text{-}430}$ and *tsi6*	This study
pETDuet-1::PA0093_282-430 ::PA0092	Co-expression vector for $tse6_{282\text{-}430}$ and *tsi6*	This study
pETDuet-1::PA0093 ::PA0092	Co-expression vector for *tse6* and *tsi6*	This study
pETDuet-1::PA0093_265-430_A268E ::PA0092	Co-expression vector for $tse6_{265\text{-}430}$ A268E point mutant and *tsi6*	This study
pETDuet-1::PA0093_265-430_L270A ::PA0092	Co-expression vector for $tse6_{265\text{-}430}$ L270A point mutant and *tsi6*	This study
pETDuet-1::PA0093_265-430_L270E ::PA0092	Co-expression vector for $tse6_{265\text{-}430}$ L270E point mutant and *tsi6*	This study
pETDuet-1::PA0093_265-	Co-expression vector for $tse6_{265\text{-}430}$ D396A	This study

430_D396A ::PA0092	point mutant and *tsi6*	
pRSFDuet::PA0094	Expression vector for *eagT6*	This study
pRSFDuet::PA0091 ::PA0094	Co-expression vector for *vgrG1* and *eagT6*	This study
pET29b::PA0092	Expression vector for *tsi6*	(Whitney et al., 2014)
pET29b::PA0092 I37M	Expression vector for *tsi6* I37M point mutant	This study
pET29b::PA4265	Expression vector for *tufA* from *P. aeruginosa*	This study
pET29b::B21_03141	Expression vector for *tufA* from *E. coli*	This study

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