Supporting Information

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SI Materials and Methods

Bacterial Strains and Growth Conditions. Strains and plasmids are listed in Table S3. Cultures were routinely grown in LB medium containing 0.5% sodium chloride at 37 °C. The following antibiotics and chemicals were added to the medium when appropriate: ampicillin (100 μg/mL), streptomycin (100 μg/mL), kanamycin (50 μg/mL), tetracycline (10 μg/mL), chloramphenicol (2.5 μg/mL for *Vibrio cholerae* and 25 μg/mL for *Escherichia coli*), and arabinose (0.1%). Mutants of *V. cholerae* and gene expression vectors were constructed as previously described (1–4). Site-directed mutagenesis was performed using the QuikChange II XL Site-Directed mutagenesis kit according to the instruction of manufacturer (Agilent). All constructs were verified by sequencing.

Construction of the Saturating Transposon Library. For library construction, we used a mariner-transposon–based vector pSAMDGm (a gift from Stephen Lory, Harvard Medical School, Boston), which is modified from the vector pSAM_Bt (5). In brief, the transposon sequence carries two recognition sites of MmeI at each end, which cuts ~20 bps downstream of the recognition site. Digestion of genomic DNA (gDNA) with MmeI thus results in DNA fragments containing the transposon sequence flanked by a short piece of gDNA (~15 bp), the sequence of which indicates the insertion location on the genome (6).

Saturating transposon library was prepared by coincubating donor *E. coli* and recipient *V. cholerae* strains on LB agar for 2 h at 37 °C. Cells were suspended in 2 mL LB and plated on LB medium containing streptomycin and gentamycin to select for transconjugants. After overnight growth at 37 °C, colonies were scraped off the plates and suspended in 3 mL of PBS buffer. Genomic DNA were extracted by adding an equal volume of lysis buffer (Phenol: Chloroform with 2 mM EDTA and 1% SDS) to cell suspension. After incubating at 65 °C for 5 min and on ice for 5 min, genomic DNA was separated from cell debris by centrifugation at 21,000 × *g* for 10 min. DNA was concentrated by precipitation with isopropanol and washed with ethanol following standard protocols. To prepare the sequencing library, purified DNA was digested with MmeI (NEB). Digested products (~1.3 Kb) were ligated to adapters containing 12 indexes for multiplexing sequencing. Ligation products were amplified by PCR with primers complementary to sequences on the adaptor and inside the transposon, respectively. The resulting PCR products were sequenced on an Illumina HiSeq2000 platform in the Biopolymer core facility at Harvard Medical School. Six independent libraries were prepared for each of wild type and the T6SS null mutant of *hcp*.

Data Analysis. Sequencing generated a large pool of reads (~50 nt), which contains the index sequence at the 5′ end, followed by ~15-bp gDNA sequence and the transposon sequence at the end. Sequencing reads were sorted based on the index and trimmed to retain only the gDNA sequence (~15 nt) by the software CLC Genomics Workbench 4.0. Reads were mapped to the reference genome *V. cholerae* N16961. The number of transposon insertion per gene is represented as the normalized RPKM value (7), which shows the relative abundance of transposon mutations in any given gene in the original transposon pool.

Western blot analysis. Western blot analysis was performed as previously described (4). Proteins were resolved in a precast 10% (wt/vol) SDS/PAGE gel (Life Technologies) and transferred to a PVDF membrane (Millipore) by electrophoresis. The membrane was then blocked in 5% (wt/vol) nonfat milk for 1 h at room temperature, and incubated with primary antibodies at 4 °C overnight. The membrane was washed three times in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH7.6), and incubated with an HRP-conjugated secondary antibody (Pierce) for 1 h at room temperature. Signals were detected using the ECL solution and ECL films (Amersham).

Protein Secretion Assay. Cultures were grown in LB medium to exponential phase (OD_{600} = 0.5). If applicable, gene expression was induced by 0.1% L-arabinose for 1 h. One milliliter of culture was centrifuged at 20,000 × *g* for 2 min and the supernatant was then filtered through a 0.2-μm filter. A mixture of 900 μL of the supernatant and 100 μL of 100% trichloroacetic acid (TCA) solution was placed on ice for 2 h, and centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatant was discarded and the pellet was washed with 1 mL of 100% acetone by centrifugation at 20,000 × *g* for 5 min. The resultant pellet was resuspended with 30 μL of SDS-loading dye. Proteins were analyzed by Western blot analysis.

T6SS-Dependent Killing Assay. Killing assay was performed as described (8). Cultures of predator and prey strains were mixed together at a ratio of 10:1, and spotted on LB medium for 3 h. Bacterial spots were then washed into 1 mL of LB and survival of prey strains was measured by serial dilution on selective medium for the growth of prey strains.

Immunoprecipitation. Exponential phase cultures (OD_{600} = 0.5) were induced with 0.1% L-arabinose for 1 h at 37 °C and harvested by centrifugation at 4,000 × *g* for 10 min. Cell pellets were resuspended in 10 mL of TBST buffer supplemented with the Halt protease inhibitor mixture (Thermo Scientific) and lysozyme (1 mg/mL). Cells were lysed by sonication and centrifuged at 10,000 × *g* for 15 min at 4 °C to remove cell debris. Anti–His-tag Dynabeads solution was prepared by mixing 0.1 mL Dynabeads (Life Technologies) with 5 μg of monoclonal anti-His antibody (Sigma). After 6 h incubation at 4 °C, the Dynabeads solution was added to the cell lysate and incubated for 2 h at 4 °C. Beads were washed 3 times with TBST buffer. Proteins were eluted with 50 μL of SDS/PAGE loading buffer, and separated on a 10% (wt/vol) SDS/PAGE gel (Life Technologies).

***Dictyostelium* Amoebae Survival Assay.** The assay was performed as described previously (9, 10). Bacterial overnight culture was diluted 10 times in PBS buffer and plated on SM/5 plate. Exponential phase culture of amoebae cells (1×10^5 CFU/mL) was collected by centrifugation and serial plated on the top of the bacterium-containing agar plates. The plates were incubated at 22 °C for 3 d, and the number of plaques formed by *Dictyostelium* was recorded.

Actin Cross-Linking Assay. Actin cross-linking was tested as described (11). To eliminate background actin cross-linking and cytotoxicity mediated by T6SS-independent toxins in *V. cholerae*, all strains tested here are in *rtxA, hapA,* and *hlyA* triple mutant background (12). One milliliter of J774 cells (~1×10^5 cells) was seeded into each well of a six-well culture plate and incubated overnight at 37 °C. Infection was performed by adding 100 μl of bacterial cells (~1×10^6 cells) to each well and incubated for 2 h. Cells were collected by scrapping and suspended in SDS-loading buffer for detection of actin by Western blot analysis.

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Table S1. Candidate immunity genes identified by Tn-seq

Gene	Gene length (bp)	Function	RPKM		*P* value
			T6SS$^+$	T6SS$^-$	
VC0821	1140	Hypothetical protein	2	53	0.012
VC1419	744	Hypothetical protein	0	71	0.001
VC1733	204	Hypothetical protein	0	126	0.003
VC1747	225	Hypothetical protein	5	210	0.003
VC1838	441	tolR membrane protein	20	179	0.012
VCA0021	729	Hypothetical protein	15	455	0.005
VCA0124	369	Hypothetical protein	0	401	0.023
VCA0431	396	Hypothetical protein	26	284	0.025

The normalized value for transposon insertions per gene is represented by RPKM. A lower RPKM value indicates there are less events of transposon insertion in the target gene.

Table S2. Comparison of *V. cholerae* effector–immunity pairs with other known T6SS immunity and effector proteins

Organism	Effector–immunity	Gene no.	Protein length (aa)	MW (kDa)	Source
P. aeruginosa	Tse1–Tsi1	PA1844-1855	154–172	16.4–18.8	(1)
	Tse2–Tsi2	PA2702-2703	158–77	17.7–8.5	
	Tse3–Tsi3	PA3484-3485	408–145	44.4–15.8	
Serratia marcescens	Ssp1–Rap1a	SM2261-2260	163–127	18.2–14.2	(2)
	Ssp2–Rap2a	SM2264-2265	158–124	17.9–13.6	
V. cholerae	TseL–TsiV1	VC1418-1419	641–247	72.2–28.5	Present study
	VasX–TsiV2	VCA0020-0021	1085–242	121–27.9	
	VgrG3–TsiV3	VCA0123-0124	1107–122	113.0–13.7	

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Table S3. Strains and plasmids in this study

Strain and plasmid	Genotype or phenotype	Source
V. cholerae		
V52	Serotype 37 clinical isolate from Sudan	(1)
T6SS mutants	Nonpolar deletion mutants of V52	(2)
V52rhh	V52 lacking *rtxA, hylE, and hapA*	(3)
*vgrG*1	V52rhh lacking *vgrG*1	(3)
tsiV1	V52 lacking genes VC1417-21	(2)
*tsiV*2	V52 lacking genes VCA0019-21	(2)
*tsiV*3	V52 lacking genes VCA0123-24	This study
pTsiV1	*tsiV1* complemented with VC1419 (TsiV1)	This study
pTsiV2	*tsiV2* complemented with VCA0021 (TsiV2)	This study
pTsiV3	*tsiV3* complemented with VCA0124 (TsiV3)	This study
tseL	V52rhh lacking VC1418 (TseL)	This study
vasX	V52rhh lacking VCA0020 (VasX)	This study
tseL & vasX	V52rhh lacking genes *tseL* and *vasX*	This study
tseL & vgrG3	V52rhh lacking genes *tseL* and *vgrG3*	This study
vasX & vgrG3	V52rhh lacking genes *vasX* and *vgrG3*	This study
tseL,vasX, & vgrG3	V52rhh triple mutant lacking *tseL*, *vasX* and *vgrG3*	This study
E. coli		
SM10 λpir	*thi thr leu tonA lac Y supE recA*::RP4-2-Tc::Mu	(4)
Plasmid		
pWM91	Suicidal conjugation vector	(5)
pSAMDGm	Transposon vector	Gift from Stephen Lory, Harvard Medical School, Boston
pBAD18V5	Expression vector with 3xV5 epitope tag at the C terminus	(6)
pTsiV1	pBAD18V5 expressing *tsiV1*	This study
pTsiV2	pBAD18V5 expressing *tsiV2*	This study
pTsiV3	pBAD18V5 expressing *tsiV3*	This study
pTseL	pBAD18V5 expressing TseL	This study
pTseL(D425A)	pBAD18V5 expressing $TseL^{D425A}$	This study
pVgrG3	pBAD24 expressing VgrG3	This study
pVgrG3(D842A)	pBAD24 expressing $VgrG3^{D842A}$	This study

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