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Supplemental Information

Independent Regulation of Type VI Secretion in *Vibrio cholerae* by TfoX and TfoY

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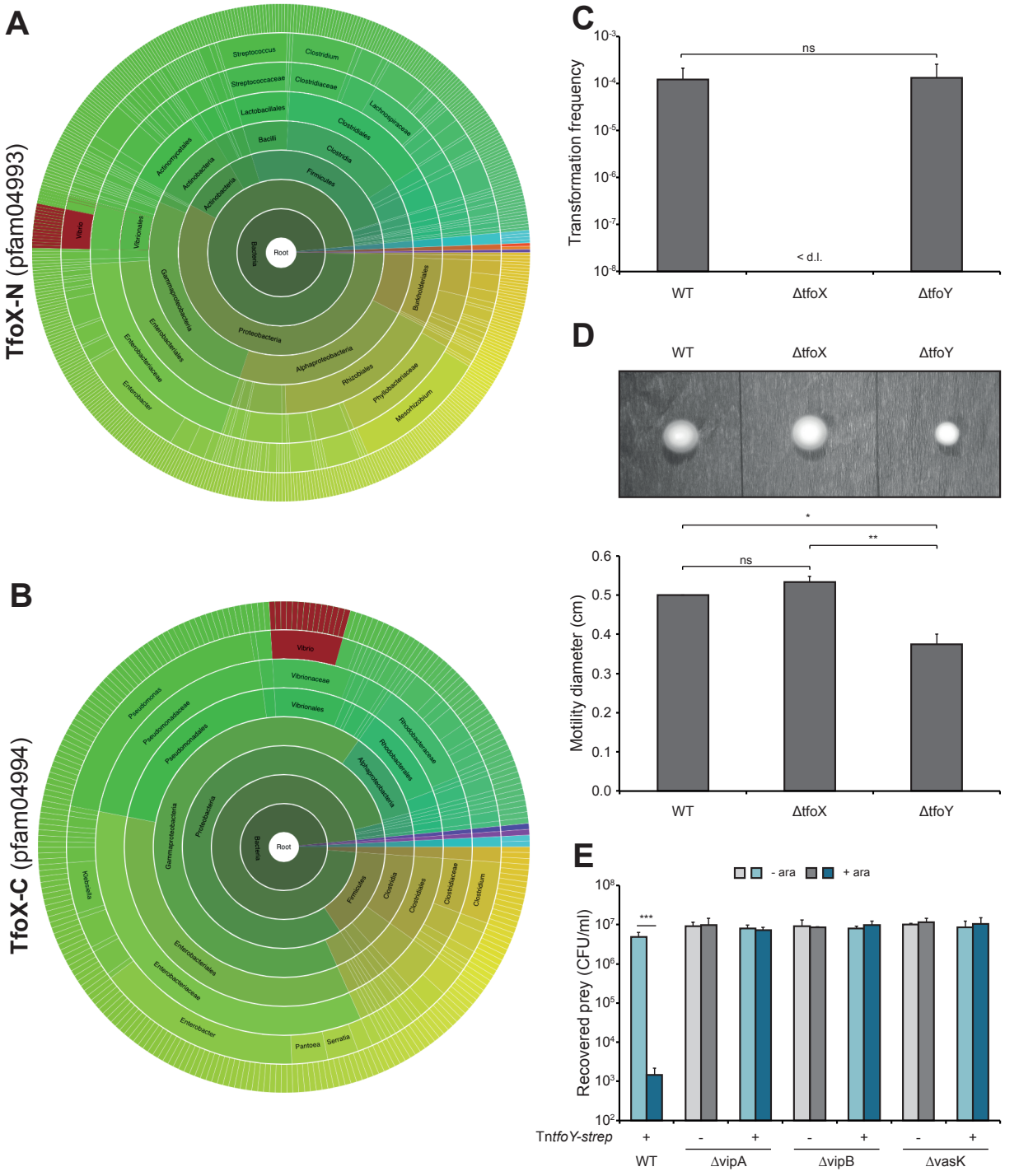

Figure S1: Kingdom-wide distribution of TfoX-like proteins, related to Figure 1. (A and B) Sunburst diagram showing the distribution of the two TfoX-like domains among different species. N-terminal domain of TfoX refers to PF04993 and the C-terminal domain to PF04994. Diagram derived from pfam.xfam.org (Pfam version 28.0). The segments are weighted by number of species. *Vibrio* species are highlighted in red. (C) TfoY is dispensable for chitin-induced natural transformation. Comparison of WT, ΔtfoX, and ΔtfoY strains with respect to natural transformability on chitin. Data are average transformation frequencies of at least three biological replicates (±SD). (D) TfoY influences bacterial motility. Comparison of WT, ΔtfoX, and ΔtfoY strain with respect to motility on soft agar. (E) Quantification of *E. coli* prey cells after co-incubation with the indicated *V. cholerae* predator cells (without (-ara) or with (+ara) arabinose). Details as in Figure 1.

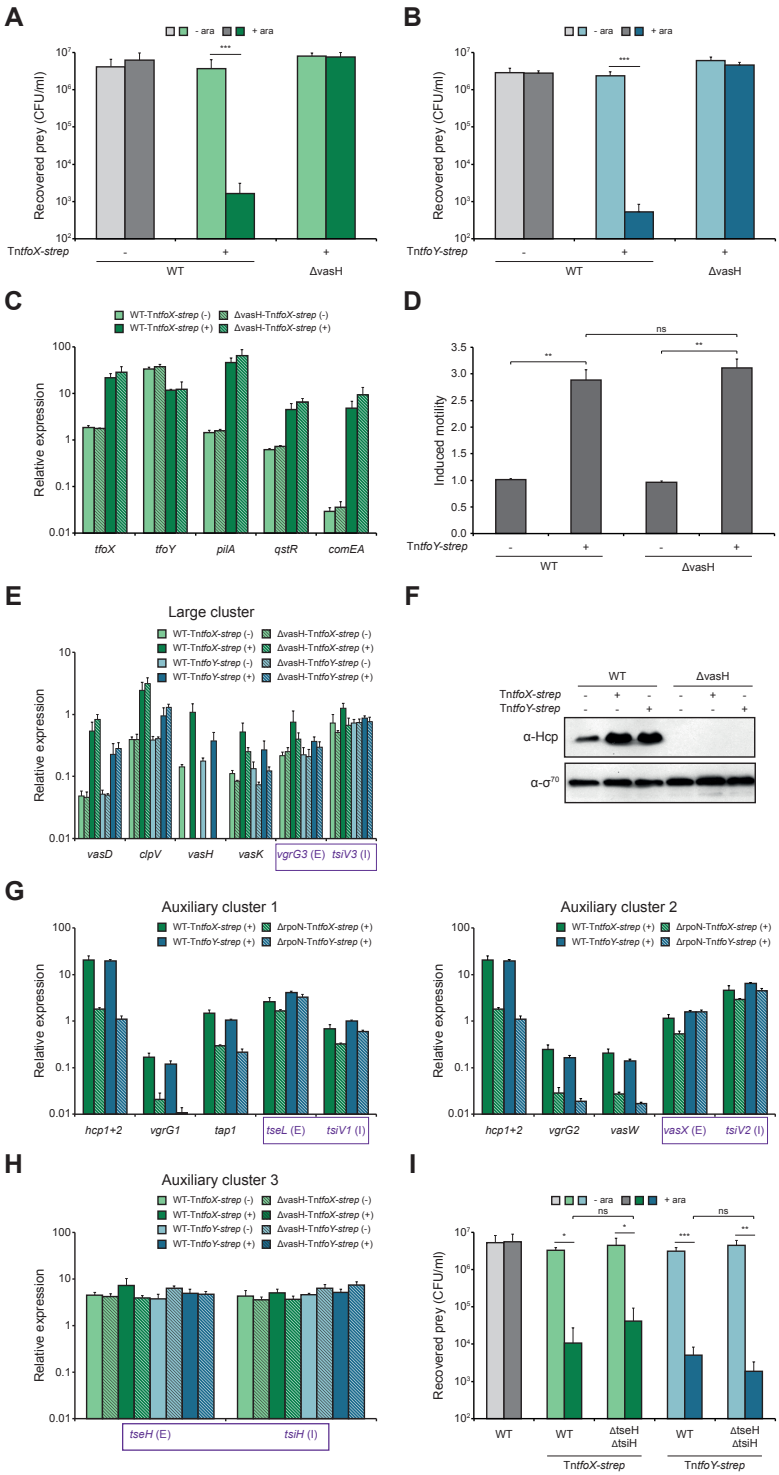

Figure S2: The contribution of VasH to TfoX- and TfoY-dependent T6SS activity, related to Figure 2. (A, B, and I) Interspecies killing assay between indicated *V. cholerae* as predator strains and *E. coli* prey. Quantification of surviving prey cells after the co-culturing with predator as described (see Figure 1). (C) TfoX-mediated competence induction does not rely on VasH. Transcript analysis of representative competence genes (*pilA, qstR, comEA*) in *V. cholerae* WT and a *vasH*-minus strain under *tfoX*-non-inducing (-) and *tfoX*-inducing (+) conditions. (D) TfoY-mediated motility enhancement occurs independently of VasH. Motility was scored for the indicated *V. cholerae* strains (as in Figure 1). (E) Transcript analysis in *V. cholerae* WT & ΔvasH. Relative gene expression analyzed by qRT-PCR as in Figure 2 but for the large T6SS cluster. (F) Detection of Hcp protein by western blotting. Cell lysates from the indicated *V. cholerae* strains grown to HCD (+ara) were analyzed for Hcp abundance. (G) Relative gene expression for the auxiliary clusters 1 and 2 comparing WT and an *rpoN*-minus strain. (H) Transcript analysis of the effector (E) and immunity (I) genes of auxiliary cluster 3. All experiments were performed at least three independent times, and error bars indicate standard deviations.

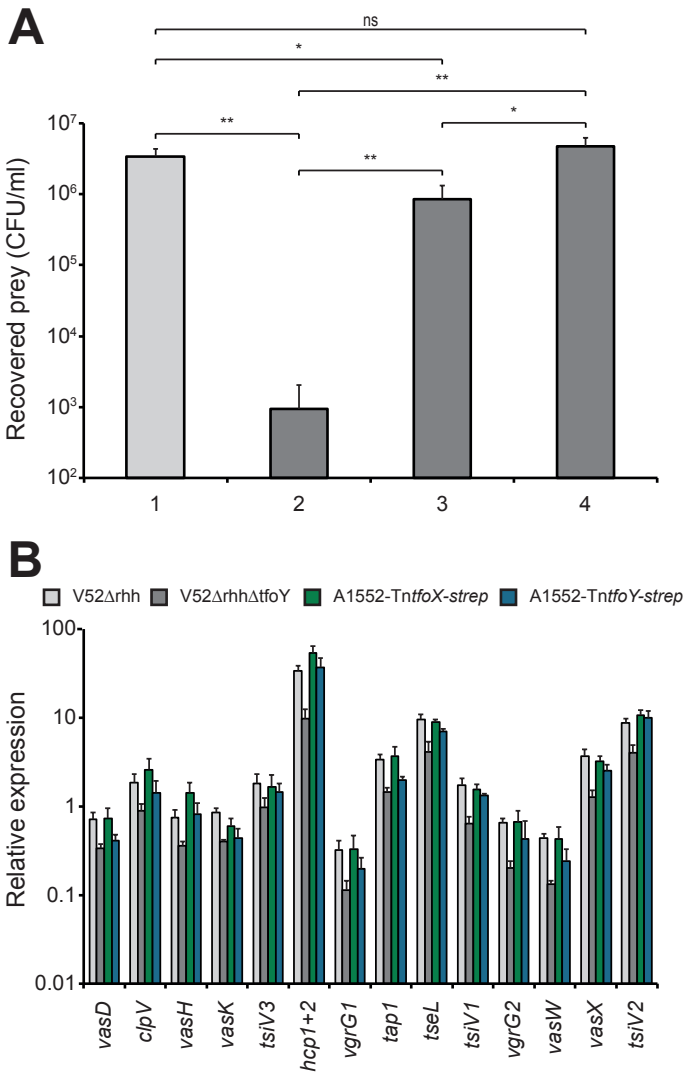

Figure S3: The constitutive T6SS activity of strain V52 depends primarily on TfoY, related to Figure 3. (A) A *tfoY*-minus mutant of V52 is severely impaired for interbacterial killing. Predator and prey were co-incubated on plain LB agar plates. *V. cholerae* predator strains tested (from 1 to 4 on X-axis): A1552, V52Δrhh, V52ΔrhhΔtfoY, and V52ΔvipB (T6SS-minus). Quantification of prey survival as described in Figure 1. (B) Relative expression of selected target genes as quantified by qRT-PCR in *V. cholerae* strains V52Δrhh, V52ΔrhhΔtfoY, A1552-Tn*tfoX*-strep and A1552-Tn*tfoY*-strep. Cells were grown to HCD in the presence of arabinose. Data are averages of three independent experiments (±SD).

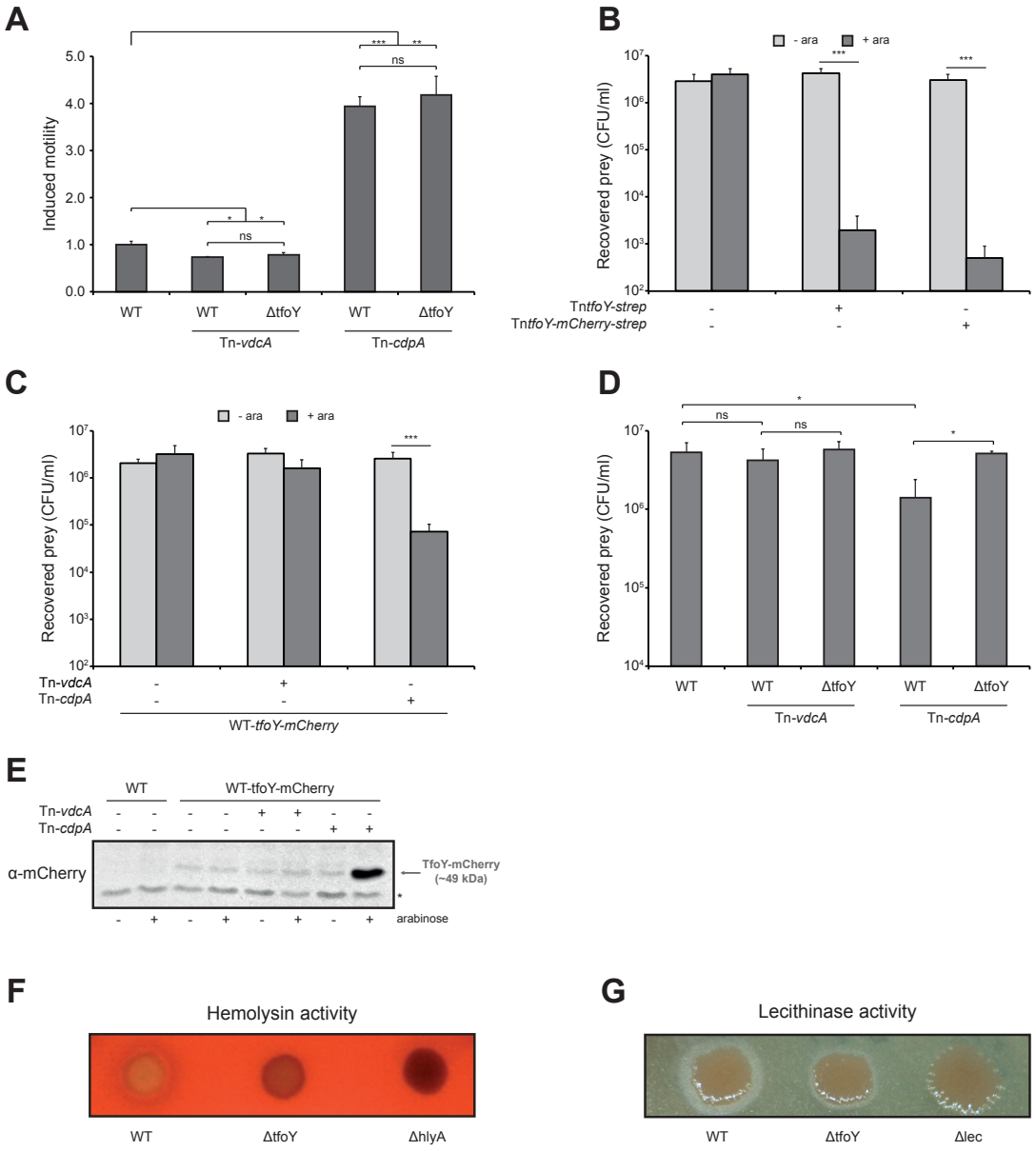

Figure S4: TfoY-mediated phenotypes and the contribution of low c-di-GMP levels to interbacterial killing, related to Figure 4. (A) Lowering the c-di-GMP levels within the cells increases bacterial motility. The indicated *V. cholerae* strains were spotted onto motility agar supplemented with arabinose to induce *vdcA* (a diguanylate cyclase) or *cdpA* (a phosphodiesterase) where indicated (Tn-*vdcA* and Tn-*cpdA*). Motility induction is indicated on the Y-axis. (B) The translational fusion between TfoY and mCherry remains full functionality. Interspecies killing assay between a WT, TfoY- (Tn*tfoY-strep*), and TfoY-mCherry-expressing (Tn*tfoY-mCherry-strep*) *V. cholerae* strain as predator and *E. coli* prey. (C) Low c-di-GMP levels lead to interbacterial killing. *E. coli* and the indicated *V. cholerae* strains (all carrying a gene encoding a functional translation fusion between *tfoY* and mCherry at the indigenous *tfoY* locus) were co-incubated on arabinose-lacking (-ara) or arabinose-containing (+ara) LB agar plates. (D) Expression of the phosphodiesterase *cdpA* results in TfoY-dependent interbacterial killing. *E. coli* and the indicated *V. cholerae* strains (WT or *tfoY*-minus mutant ΔtfoY) expressing a diguanylate cyclase (Tn-*vdcA*) or a phosphodiesterase (Tn-*cdpA*) were co-incubated on arabinose-containing (+ara) LB agar plates. Details as in Figure 1. (E) Detection of TfoY-mCherry produced by c-di-GMP phosphodiesterase-expressing cells. *VdcA* or *cdpA* expression was induced by arabinose where indicated. The WT strain, which lacks the *tfoY-mCherry* fusion construct, served as negative control. Western blot analysis was performed as described for Figure 3C except that mCherry-specific antibodies were utilized. A nonspecific cross-reaction (marked with an asterisk) serves as a loading control. (F) Hemolytic activity of the indicated strains was tested on blood agar plates. The WT and an *hlyA*-mutant served as the positive and negative control, respectively. (G) Activity of the lecithinase (thermolabile hemolysin) was assessed on egg yolk plates. The WT and a *lec*-minus strain served as controls.

Table S1: RNA-seq expression data for the T6SS gene clusters in WT compared to TfoX[#]- and TfoY-induced cells.

cluster names	gene names	ID (locus tag; Heidelberg *et al*., 2000)	A1552 (WT) +ara_Exp1	A1552 (WT) +ara_Exp2	A1552 (WT) +ara_Exp3	AVG A1552 (WT) +ara	A1552-TntfoX+ara_ Exp1 (Borgeaud *et al*., 2015)	A1552-TntfoX+ara_ Exp2 (Borgeaud *et al*., 2015)	A1552-TntfoX+ara_ Exp3 (Borgeaud *et al*., 2015)	AVG A1552-TntfoX+ara (Borgeaud *et al*., 2015)	TfoX-induced (TfoX+ / WT)	A1552-TntfoY +ara_Exp1	A1552-TntfoY +ara_Exp2	A1552-TntfoY +ara_Exp3	AVG A1552-TntfoY +ara	TfoY-induced (TfoY+ / WT)
	tfoX	**VC1153**	8128.63	9285.04	10233.82	9215.83	29170.99	25425.36	25921.82	26839.39	2.91	5798.01	6251.69	6184.87	6078.19	0.66
	tfoY	**VC1722**	7491.01	6985.98	7931.44	7469.48	996.62	1176.54	1354.46	1175.87	0.16	31298.07	33495.54	35071.18	33288.26	4.46
Auxiliary cluster 1	*hcp1*	VC1415	120.58	137.72	86.41	114.90	4772.38	6025.73	3508.95	4769.02	41.51	3356.54	3977.41	3539.18	3624.38	31.54
	vgrG1	VC1416	108.31	106.99	103.51	106.27	1263.24	1134.86	1042.16	1146.75	10.79	620.53	761.19	710.67	697.46	6.56
	tap1	VC1417	124.66	97.88	118.81	113.78	585.63	471.75	444.47	500.62	4.40	470.19	491.62	527.85	496.56	4.36
	tseL	VC1418	488.43	503.06	586.85	526.11	2287.81	2180.67	1810.62	2093.03	3.98	3503.05	3966.05	4746.79	4071.96	7.74
	tsiV1	VC1419	92.99	124.06	126.01	114.35	654.32	921.72	658.51	744.85	6.51	794.65	958.46	1323.49	1025.53	8.97
Auxiliary cluster 2	*hcp2*	VCA0017	135.90	159.34	87.31	127.52	4629.17	5281.16	3434.10	4448.14	34.88	3432.48	3975.34	3540.46	3649.43	28.62
	vgrG2	VCA0018	18.39	19.35	22.50	20.08	482.01	404.49	449.15	445.22	22.17	203.26	231.35	140.33	191.65	9.54
	vasW	VCA0019	34.74	35.28	27.00	32.34	271.28	338.18	290.07	299.84	9.27	648.14	678.57	687.49	671.40	20.76
	vasX	VCA0020	346.40	359.66	303.32	336.46	1744.09	1784.70	1512.36	1680.38	4.99	2604.85	2566.57	2630.24	2600.55	7.73
	tsiV2	VCA0021	271.81	319.82	263.72	285.12	684.60	862.04	695.94	747.53	2.62	1007.12	1128.88	1203.76	1113.25	3.90
	PAAR1	VCA0105	83.79	78.53	129.61	97.31	357.43	231.14	266.68	285.08	2.93	258.49	296.42	239.46	264.79	2.72
		VCA0106	91.96	100.16	144.01	112.04	618.23	434.81	402.36	485.13	4.33	316.79	369.75	418.42	368.32	3.29
Large/ major cluster	*vipA*	VCA0107	50.07	59.18	47.70	52.32	1341.25	1093.18	1058.53	1164.32	22.25	418.03	515.38	525.28	486.23	9.29
	vipB	VCA0108	180.86	187.80	180.91	183.19	3384.56	2519.80	2610.66	2838.34	15.49	1337.71	1502.76	1396.87	1412.45	7.71
		VCA0109	56.20	76.26	46.80	59.75	1068.81	747.41	855.02	890.41	14.90	493.20	572.18	431.29	498.89	8.35
	vasA	VCA0110	120.58	118.37	123.31	120.75	1586.91	1307.26	1319.37	1404.51	11.63	730.98	686.83	757.02	724.94	6.00
	vasB	VCA0111	33.72	35.28	29.70	32.90	626.38	591.11	487.74	568.41	17.28	257.72	322.24	294.82	291.60	8.86
	fha	VCA0112	84.81	87.64	81.91	84.79	1009.43	842.14	884.26	911.94	10.76	520.82	549.46	608.96	559.75	6.60
	vasD	VCA0113	75.62	66.01	79.21	73.61	664.80	632.79	542.72	613.44	8.33	418.80	466.84	442.88	442.84	6.02
	vasE	VCA0114	87.88	96.74	87.31	90.64	839.44	1128.23	935.72	967.80	10.68	579.88	679.60	576.77	612.08	6.75
	vasF	VCA0115	43.94	51.22	39.60	44.92	465.71	521.01	479.56	488.76	10.88	312.18	327.41	293.54	311.04	6.92
	clpV	VCA0116	289.18	332.34	274.52	298.68	1832.57	2443.07	2014.14	2096.59	7.02	1326.97	1808.48	1462.53	1532.66	5.13
	vasH	VCA0117	197.21	253.81	244.82	231.95	1305.16	1833.96	1505.34	1548.15	6.67	1062.34	1369.53	1180.58	1204.15	5.19
	vasI	VCA0118	39.85	46.66	29.70	38.74	310.86	440.49	333.35	361.57	9.33	233.95	235.48	215.00	228.14	5.89
	vasJ	VCA0119	139.99	166.17	117.91	141.36	773.08	1092.23	895.95	920.42	6.51	741.72	941.94	749.29	810.98	5.74
	vasK	VCA0120	525.22	680.61	460.84	555.56	2522.99	3768.33	2609.49	2966.94	5.34	2324.88	3200.72	2822.07	2782.56	5.01
	vasL	VCA0121	330.05	374.45	288.02	330.84	936.08	1589.56	1180.18	1235.27	3.73	1122.17	1591.58	1350.53	1354.76	4.09
	vasM	VCA0122	38.83	34.14	33.30	35.43	64.04	92.83	101.76	86.21	2.43	92.81	124.97	124.88	114.22	3.22
	vgrG3	VCA0123	790.89	931.01	806.46	842.79	1636.97	2488.54	1864.42	1996.65	2.37	1963.61	2550.04	2471.89	2328.51	2.76
	tsiV3	VCA0124	345.38	586.15	477.94	469.82	826.64	1108.33	800.04	911.67	1.94	715.64	1093.76	1084.03	964.48	2.05
Auxiliary cluster 3	*PAAR2*	VCA0284	157.36	166.17	284.42	202.65	235.18	222.61	216.39	224.73	1.11	293.77	361.49	379.80	345.02	1.70
	tseH	VCA0285	182.91	146.82	275.42	201.72	308.53	294.61	271.36	291.50	1.45	423.40	424.49	561.32	469.74	2.33
	tsiH	VCA0286	161.45	129.75	221.42	170.87	239.84	306.92	232.76	259.84	1.52	505.48	527.77	690.07	574.44	3.36

TfoX-induced data are from a previous study (Borgeaud et al., 2015).

Table S2: Bacterial cytotoxicity against the amoeba *Dictyostelium discoideum*

	Numbers of plaques[#] (±SD)	Statistics compared to strain V52Δrhh	Relative population size compared to *Klebsiella* negative control
V52Δrhh	17 (± 11)		10.2%
V52ΔrhhΔtfoY	159 (± 25)	** (p = 0.00619)	95.2%
Klebsiella sp.	167 (± 18)	** (p = 0.00129)	100%

Average number of plaques from three independent experiments with three replicates each ± standard deviation (SD).

Table S3: *V. cholerae* strains and plasmids used in this study

Strains or Plasmid	Genotype*/description	Internal strain No	Reference
A1552 (WT)	Wild-type, O1 El Tor Inaba; Rif^R	MB_1	(Yildiz and Schoolnik, 1998)
A1552-Tn*tfoX-strep*	A1552 containing mini-Tn7-*araC*-P_{BAD}-*tfoX-strep*; Rif^R, $Gent^R$	MB_3420	This study
A1552-Tn*tfoY*	A1552 containing mini-Tn7-*araC*-P_{BAD}-*tfoY*; Rif^R, $Gent^R$	MB_2979	This study
A1552-Tn*tfoY-strep*	A1552 containing mini-Tn7-*araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_2978	This study
A1552-Tn*tfoY-mCherry-strep*	A1552 containing mini-Tn7- *araC*-P_{BAD}-*tfoY-mCherry-strep*; Rif^R, $Gent^R$	MB_4322	This study
A1552-Tn-*vdcA*	A1552 containing mini-Tn7-*araC*-P_{BAD}-*vdcA*; Rif^R, $Gent^R$	MB_2947	This study
A1552-Tn-*cdpA*	A1552 containing mini-Tn7-*araC*-P_{BAD}-*cdpA*; Rif^R, $Gent^R$	MB_2948	This study
A1552ΔhapR	A1552ΔVC0583; Rif^R	MB_3	(Meibom et al., 2005)
A1552ΔhapR-Tn*tfoY-strep*	A1552ΔhapR containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_4267	This study
A1552ΔhlyA	A1552 deleted for *VCA0219* (TransFLP); Rif^R	MB_3935	This study
A1552Δlec	A1552 deleted for *VCA0218* (TransFLP); Rif^R	MB_4189	This study
A1552ΔqstR	A1552ΔVC0396; Rif^R	MB_600	(Lo Scrudato and Blokesch, 2013)
A1552ΔqstR-Tn*tfoY-strep*	A1552ΔqstR containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_3772	This study
A1552ΔrpoN	A1552 deleted for *VC2529* (TransFLP); Rif^R	MB_4034	This study
A1552ΔrpoN-Tn*tfoX-strep*	A1552ΔrpoN (TransFLP) containing mini-Tn7- *araC*-P_{BAD}-*tfoX-strep*; Rif^R, $Gent^R$	MB_4088	This study
A1552ΔrpoN-Tn*tfoY-strep*	A1552ΔrpoN (TransFLP) containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_4089	This study
A1552ΔtfoX	A1552ΔtfoX (TransFLP); Rif^R	MB_1447	(Borgeaud et al., 2015)
A1552ΔtfoX-Tn*tfoY-strep*	A1552ΔtfoX (TransFLP) containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_3418	This study
A1552ΔtfoY	A1552ΔVC1722 (deleted using suicide plasmid pGP704-28-SacB-Δ*tfoY*); Rif^R	MB_828	This study
A1552ΔtfoY-Tn*tfoX-strep*	A1552ΔtfoY containing mini-Tn7- *araC*-P_{BAD}-*tfoX-strep*; Rif^R, $Gent^R$	MB_3419	This study
A1552ΔtfoY-Tn-*vdcA*	A1552ΔtfoY containing mini-Tn7- *araC*-P_{BAD}-*vdcA*; Rif^R, $Gent^R$	MB_3945	This study
A1552ΔtfoY-Tn-*cdpA*	A1552ΔtfoY containing mini-Tn7- *araC*-P_{BAD}-*cdpA*; Rif^R, $Gent^R$	MB_3946	This study
A1552ΔtseHΔtsiH	A1552 deleted for *VCA0285* and *VCA0286* (TransFLP); Rif^R	MB_4131	This study
A1552ΔtseHΔtsiH-Tn*tfoX-strep*	A1552ΔtseHΔtsiH (TransFLP) containing mini-Tn7- *araC*-P_{BAD}-*tfoX-strep*; Rif^R, $Gent^R$	MB_4185	This study
A1552ΔtseHΔtsiH-Tn*tfoY-strep*	A1552ΔtseHΔtsiH (TransFLP) containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_4186	This study
A1552ΔvasH	A1552 deleted for *VCA0117* (TransFLP); Rif^R	MB_3928	This study
A1552ΔvasH-Tn*tfoX-strep*	A1552ΔvasH (TransFLP) containing mini-Tn7- *araC*-P_{BAD}-*tfoX-strep* -strep; Rif^R, $Gent^R$	MB_3936	This study
A1552ΔvasH-Tn*tfoY-strep*	A1552ΔvasH (TransFLP) containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_3937	This study
A1552ΔvasK	A1552ΔVCA0120; Rif^R	MB_585	(Borgeaud et al., 2015)

Strains or Plasmid	Genotype*/description	Internal strain No	Reference
A1552ΔvasK-Tn*tfoY-strep*	A1552ΔvasK containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_3578	This study
A1552ΔvipA	A1552ΔVCA0107 (TransFLP); Rif^R	MB_3042	(Borgeaud et al., 2015)
A1552ΔvipA-Tn*tfoY-strep*	A1552ΔvipA containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_3580	This study
A1552ΔvipB	A1552ΔVCA0108; Rif^R	MB_598	(Borgeaud et al., 2015)
A1552ΔvipB-Tn*tfoY-strep*	A1552ΔvipB containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_3579	This study
A1552-tfoY-mCherry	A1552 carrying *tfoY-mCherry* translational fusion (TransFLP); Rif^R	MB_4262	This study
A1552-tfoY-mCherry-Tn-*vdcA*	A1552-tfoY-mCherry containing mini-Tn7- *araC*-P_{BAD}-*vdcA*; Rif^R, $Gent^R$	MB_4324	This study
A1552-tfoY-mCherry-Tn-*cdpA*	A1552-tfoY-mCherry containing mini-Tn7- *araC*-P_{BAD}-*cdpA*; Rif^R, $Gent^R$	MB_4325	This study
A1552-vipA-sfGFPv2	A1552 carrying *vipA-sfgfp* translational fusion (TransFLP), as previously described (Borgeaud et al., 2015); this fusion version (v2) has 3 bp of the linker region removed; Rif^R	MB_3909	This study
A1552-vipA-sfGFPv2-Tn*tfoX-strep*	A1552-vipA-sfGFPv2 containing mini-Tn7- *araC*-P_{BAD}-*tfoX-strep*; Rif^R, $Gent^R$	MB_3961	This study
A1552-vipA-sfGFPv2-Tn*tfoY-strep*	A1552-vipA-sfGFPv2 containing mini-Tn7 *araC*-P_{BAD}-*tfoY-strep* Rif^R, $Gent^R$	MB_3962	This study
A1552ΔhapR-vipA-sfGFPv2	A1552-vipA-sfGFPv2 (TransFLP) ΔVC0583 (deleted using suicide plasmid pGP704-28-SacB-Δ*hapR*); Rif^R	MB_4217	This study
A1552ΔhapR-vipA-sfGFPv2-Tn*tfoX-strep*	A1552ΔhapR-vipA-sfGFPv2 containing mini-Tn7- *araC*-P_{BAD}-*tfoX-strep*; Rif^R, $Gent^R$	MB_4261	This study
A1552ΔhapR-vipA-sfGFPv2-Tn*tfoY-strep*	A1552ΔhapR-vipA-sfGFPv2 containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Rif^R, $Gent^R$	MB_4260	This study
SA5YΔvipA-*dsRED*	SA5YΔvipA-*dsRED;* Kan^R, $Gent^R$	MB_3052	(Borgeaud et al., 2015)
V52Δrhh	V52Δrhh; Str^R	MB_3778	(Basler et al., 2012)
V52Δrhh-Tn*tfoY-strep*	V52Δrhh containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Str^R, $Gent^R$	MB_4209	This study
V52ΔrhhΔtfoY	V52ΔrhhΔVC1722 (deleted using suicide plasmid pGP704-28-SacB-Δ*tfoY*); ; Str^R	MB_4179	This study
V52ΔrhhΔtfoY-Tn*tfoY-strep*	V52ΔrhhΔtfoY containing mini-Tn7- *araC*-P_{BAD}-*tfoY-strep*; Str^R, $Gent^R$	MB_4211	This study
V52ΔrhhΔtfoX	V52ΔrhhΔVC1153 (deleted using suicide plasmid pGP704-28-SacB-Δ*tfoX*); Str^R	MB_4383	This study
***E. coli* & others**			
SM10λpir	thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu, Kmr (λpir); Kan^R	MB_647	(Simon et al., 1983)
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araΔ139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1λ^-	MB_741	Invitrogen
TOP10-TnKan	TOP10 containing mini-Tn7-*aph* (Kan^R); Str^R, Kan^R, $Gent^R$	MB_4119	This study
DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG φ80lacZΔM15 Δ(lacZYA-argF) U169 hsdR17 (r_K^- m_K^+) phoA, λ-	MB_736	(Yanisch-Perron et al., 1985)
S17-1λpir	Tpr Smr recA thi pro hsdR2M1 RP4:2-Tc:Mu:Kmr Tn7 (λpir); Str^R	MB_648	(Simon et al., 1983)

Strains or Plasmid	Genotype*/description	Internal strain No	Reference
Klebsiella sp.	*Klebsiella* species; *gfp*$^{+}$	MB_4331	(Benghezal et al., 2006) (P. Cosson, Geneva)
Plasmids			
pBAD/myc-HisA	pBR322-derived expression vector; *araBAD* promoter (P_{BAD}); Amp^{R}	MB_24	Invitrogen
pBAD-*tfoX-strep*	*tfoX* in pBAD/Myc-HisA with C-terminal *Strep*-tagII, arabinose inducible; Amp^{R}	MB_3616	This study
pBAD-*tfoY*	*tfoY* in pBAD/Myc-HisA without tag, arabinose inducible; Amp^{R}	MB_768	This study
pBAD-*tfoY-strep*	*tfoY* in pBAD/Myc-HisA with C-terminal *Strep*-tagII, arabinose inducible; Amp^{R}	MB_2945	This study
pBAD-*tfoY-strep*-CSmaI	pBAD-*tfoY-strep* including SmaI site between tfoY gene and *Strep*-tagII; Amp^{R}	MB_4385	This study
pBAD-*tfoY-mCherry*-strep	*tfoY-mCherry* (translational fusion) in pBAD/Myc-HisA with C-terminal *Strep*-tagII, arabinose inducible; Amp^{R}	MB_4386	This study
pBAD-*vdcA*	*vdcA* in pBAD/Myc-HisA without tag, arabinose inducible; Amp^{R}	MB_2066	This study
pBAD-*cdpA*	*cdpA* in pBAD/Myc-HisA without tag, arabinose inducible; Amp^{R}	MB_2065	This study
pGP704-mTn7-minus SacI	pGP704 with mini-*Tn*7; Amp^{R}, Gent^{R}	MB_645	(Müller et al., 2007)
pGP704-mTn*tfoX-strep*	pGP704 with mini-Tn7 carrying *araC* and P_{BAD}-driven *tfoX-strep*; Amp^{R}, Gent^{R}	MB_3664	This study
pGP704-mTn*tfoY*	pGP704 with mini-Tn7 carrying *araC* and P_{BAD}-driven *tfoY*; Amp^{R}, Gent^{R}	MB_2953	This study
pGP704-mTn*tfoY-strep*	pGP704 with mini-Tn7 carrying *araC* and P_{BAD}-driven *tfoY-strep*; Amp^{R}, Gent^{R}	MB_2941	This study
pGP704-mTn*tfoY-mCherry-strep*	pGP704 with mini-Tn7 carrying *araC* and P_{BAD}-driven *tfoY-mCherry-strep*; Amp^{R}, Gent^{R}	MB_4387	This study
pGP704-mTn-*vdcA*	pGP704 with mini-Tn7 carrying *araC* and P_{BAD}-driven *vdcA*; Amp^{R}, Gent^{R}	MB_2943	This study
pGP704-mTn-*cdpA*	pGP704 with mini-Tn7 carrying *araC* and P_{BAD}-driven *cdpA*; Amp^{R}, Gent^{R}	MB_2944	This study
pGP704-TnKan	pGP704 with mini-Tn7 carrying *aph* (Kan^{R}) gene; Amp^{R}, Gent^{R}, Kan^{R}	MB_4117	This study
pUX-BF-13	*ori*R6K, helper plasmid with Tn7 transposition function; Amp^{R}	MB_457	(Bao et al., 1991)
pGP704-Sac28	Suicide vector, *ori*R6K, *sacB*; Amp^{R}	MB_649	(Meibom et al., 2004)
pGP704-28-SacB-Δ*tfoY*	pGP704-Sac28 with gene fragment resulting in a 180 bp deletion within *VC1722*; Amp^{R}	MB_1133	This study
p28-hapR (pGP704-28-SacB-Δ*hapR*)	pGP704-Sac28 with gene fragment resulting in a deletion from amino acid 10 downwards of *hapR;* Amp^{R}	MB_1106	(Meibom et al., 2005)
p28-tfoX (pGP704-28-SacB-Δ*tfoX*)	pGP704-Sac28 with a gene fragment resulting in a deletion of amino acids 6-196 of TfoX; Amp^{R}	MB_1013	This study
pBR-FRT-Kan-FRT2	pBR322 derivative containing improved FRT-*aph*-FRT cassette, used as template for TransFLP; Amp^{R}, Kan^{R}	MB_3782	This study
pBR-FRT-Cat-FRT2	pBR322 derivative containing improved FRT-*cat*-FRT cassette, used as template for TransFLP; Amp^{R}, Cm^{R}	MB_3783	This study

*VC numbers according to (Heidelberg et al., 2000).

EXTENDED EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, and growth conditions

V. cholerae strains and plasmids are listed in Table S3. *Escherichia coli* strains DH5α (Yanisch-Perron et al., 1985), TOP10 (Invitrogen), and S17-1λpir (Simon et al., 1983) were used for cloning purposes and served as donors in bacterial mating experiments, respectively. A V52Δrhh (Δ*rtxA* Δ*hlyA* Δ*hapA*) strain served as the parental strain for T6SS activity and amoebal killing (see below), in accordance with previous protocols (Bachmann et al., 2015; Miyata et al., 2011).

V. cholerae and *E. coli* strains were grown aerobically in LB medium or on LB agar plates at 30°C or 37°C unless otherwise stated. LB motility soft agar plates contained reduced agar concentrations (0.3%) compared to standard LB agar plates (1.5%). When required, arabinose (for the expression of *tfoX*, *tfoY, tfoY-mCherry, vdcA*, and *cpdA* under the control of the P_{BAD} promoter) or antibiotics were added to the growth medium at the following concentrations: L-arabinose 0.02% or 0.2%, ampicillin 50 μg/ml or 100 μg/ml, kanamycin 75 μg/ml, streptomycin 100 μg/ml, chloramphenicol 2.5 μg/ml, and gentamicin 50 μg/ml. For *E. coli* counter-selection after tri-parental mating with *V. cholerae,* Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates were used and prepared following the manufactures' instructions (Sigma-Aldrich/Fluka, Buchs, Switzerland). Half-concentration defined artificial seawater (0.5x DASW; (Meibom et al., 2005)) was used for chitin-induced natural transformation experiments.

Genetic engineering of strains and plasmids

All DNA manipulations were performed according to standard molecular biology protocols. Enzymes were purchased from the following companies and used as recommended by the manufacturer: Pwo polymerase (Roche), Taq polymerase (Promega), and restriction enzymes (New England Biolabs). Genetically engineered bacterial strains and plasmids were screened by colony PCR followed by Sanger sequencing (Microsynth, Switzerland).

To genetically modify *V. cholerae* strains, a gene disruption method based on the counter-selectable suicide plasmid pGP704-Sac28 (Meibom et al., 2004) or a transformation-based genetic engineering method (TransFLP; (Blokesch, 2012; Borgeaud and Blokesch, 2013; De Souza Silva and Blokesch, 2010)) was utilized. The latter technique was also used to replace the *tfoY* gene of *V. cholerae* A1552 with a translational *tfoY-mCherry* fusion allele at the gene's native locus. The plasmids pBR-FRT-Kan-FRT2 and pBR-FRT-Cat-FRT2 served as templates for the addition of the flippable antibiotics cassette.

Plasmid pBAD-*tfoX-strep* was constructed by amplification of the *tfoX* gene with *Strep-tagII*-encoding primers using the genomic DNA (gDNA) of *V. cholerae* strain A1552 as the template. The restriction enzyme-digested PCR product was cloned into the appropriately digested plasmid pBAD/MycHisA (Table S3). The *tfoY* gene was amplified using gDNA from *V. cholerae* strain A1552 and cloned after restriction enzyme digestion into the plasmid pBAD/MycHisA, resulting in plasmid pBAD-*tfoY* (Table S3). The same strategy was employed to obtain plasmids pBAD-*vdcA* and pBAD-*cdpA* (Table S3). The *Strep-tagII*-encoding sequence was added to *tfoY* by inverse PCR using overlapping oligonucleotides. The template was subsequently digested by *Dpn*I, and the non-digested PCR fragment was column-purified and used directly to transform chemically competent *E. coli* cells.

The plasmid pBAD-*tfoY-mCherry-strep* was constructed in two steps. First, a *Sma*I restriction site was introduced in-between the *tfoY* open reading frame and the *Strep-tagII* by inverse PCR on plasmid pBAD-*tfoY-strep,* as described above, resulting in plasmid pBAD-*tfoY-strep*-CSmaI. Subsequently, the mCherry-encoding gene was amplified using phosphorylated primers and cloned into the *Sma*I-digested pBAD-*tfoY-strep*-CSmaI plasmid.

The fragments containing *araC*, the arabinose-inducible promoter P_{BAD}, and the *tfoX-strep*, *tfoY-strep, tfoY-mCherry-strep*, *vdcA* or *cdpA* gene were amplified from the corresponding pBAD plasmids (Table S3) and cloned into the mini-Tn7-containing delivery plasmid (Table S3). For the insertion of this mini-Tn7 transposon into the *V. cholerae* chromosome, a triparental mating strategy was employed (Bao et al., 1991). The donor plasmids are indicated in Table S3.

Natural transformation assays

The natural transformability of *V. cholerae* strains grown on chitin surfaces was determined as previously described (De Souza Silva and Blokesch, 2010; Marvig and Blokesch, 2010). To test for chitin-independent transformation, the strains carried an arabinose-inducible copy of *tfoX-strep* (e.g., including the *Strep*-tagII-encoding sequence) or *tfoY-strep* on the chromosome. The assay was performed as previously described (Lo Scrudato and Blokesch, 2012). The gDNA of A1552-lacZ-Kan (Marvig and Blokesch, 2010) served as the transforming material in all transformation assays. Transformation frequencies were calculated as the number of transformants divided by the total number of colony-forming units (CFU). At least three independent biological replicates were performed for

each experiment, and averages of all experiments are indicated in the figure (±SD). Statistical analyses of transformation frequencies were performed on log-transformed data using a two-tailed Student's *t*-test. For values below the detection limit, the detection limit value was utilized for statistical calculations.

Interbacterial killing assay

The interbacterial killing assay was performed following a previously established protocol (Borgeaud et al., 2015). Briefly, the predatory *V. cholerae* strain and the *E. coli* prey were mixed at a 10:1 ratio and spotted onto membrane filters on pre-warmed LB agar plates (± 0.2% ara). After incubation at 37°C for 4 h, the bacteria were resuspended from the filters, and serial dilutions were spotted onto plain LB (growth control) and antibiotic-containing LB agar plates (to select for the respective *E. coli* prey strains) to enumerate colony-forming units (CFU/ml). At least three biological experiments were performed, and averages of these independent replicates are given throughout the manuscript. Statistically significant differences were determined by the two-tailed Student's *t*-test on log-transformed data. In case the *E. coli* CFUs were below the detection limit of 200 CFU/ml, the value was set to the detection limit to allow statistical analysis.

Motility assay

To assess the motility of *V. cholerae,* 2 µl of the relevant overnight culture was carefully spotted onto freshly prepared LB motility agar plates and incubated at room temperature for 24 h. The next day, the swarming diameter was scored. The motility induction was calculated by dividing the swarming diameter under induced versus uninduced conditions. All experiments were repeated several times independently ($n \geq 3$), and averages of all experiments (± standard deviation) are provided. A two-tailed Student's *t*-test was performed for statistical analyses.

Gene expression analysis by qRT-PCR

Quantitative reverse transcription PCR (qRT-PCR)-based transcript scoring in *V. cholerae* was performed following a previously published protocol (Lo Scrudato and Blokesch, 2012). Averages of at least three biologically independent experiments (± standard deviation) are provided.

SDS-PAGE and Western Blotting

For the preparation of cell lysates, bacteria were cultivated in LB medium without or with arabinose, as indicated. After harvesting, the bacterial cells were resuspended in an appropriate volume of Laemmli buffer to adjust for the total number of bacteria (based on measurement of the optical density at 600 nm; OD_{600}) and boiled for 15 minutes. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and subjected to western blotting as previously described (Lo Scrudato and Blokesch, 2012). Primary antibodies against Hcp (see below), *E. coli* Sigma 70 (BioLegend, USA distributed via Brunschwig, Switzerland), or mCherry (BioVision, USA distributed via LubioScience, Switzerland) were diluted at 1:5'000. Goat anti-rabbit horseradish peroxidase (HRP) (diluted 1:20'000; Sigma-Aldrich, Switzerland) and goat anti-mouse HRP (diluted 1:5'000, Sigma-Aldrich, Switzerland) were used as secondary antibodies. Lumi-Light^{PLUS} western blotting substrate (Roche, Switzerland) was used as the HRP substrate and the signals were detected on chemiluminescence-detecting films (Amersham Hyperfilm ECL, GE Healthcare).

Generation of antibodies against Hcp

Anti-Hcp antibodies were raised in rabbits against synthetic peptides (Eurogentec, Belgium). To exclude potential cross-reactions with proteins migrating towards the same position as the target protein, the antibody was tested against the *hcp1 hcp2* double knockout strain using western blotting (data not shown).

Epifluorescence microscopy and image analysis

Wide-field microscopy images were acquired using an epifluorescence microscope (Zeiss Axio Imager M2; details are provided elsewhere (Lo Scrudato and Blokesch, 2012)). The bacterial cultures were grown in LB medium (±ara) and then mounted onto agarose pads. Image analysis and processing were performed using the Zeiss AxioVision software, ImageJ, and Adobe Illustrator. For comparisons between different culture conditions (e.g., WT versus *hapR*-minus strains grown in the absence or presence of inducible *tfoX* or *tfoY*), the images were acquired using the exact same exposure time. However, the brightness was decreased for the TfoX-induced vipA-sfGFP signal for a better representation. For intraspecies killing, the two strains (predator and non-immune prey) were mixed at a ratio of 1:1 before being applied to an agarose pad. The mixed communities were incubated for 2 h at 24°C before imaging.

***Dictyostelium discoideum* plaque assay**

To determine the cytotoxicity of *V. cholerae* strain V52 (V52Δrhh) toward *D. discoideum*, an amoebal plaque assay was performed following a previously described protocol (Pukatzki et al., 2006) with slight modifications. Briefly, bacteria were cultured overnight in LB medium at 30°C and harvested by centrifugation. The cell pellet was resuspended in SorC buffer and diluted to an OD_{600} of 5.5. *D. discoideum* cells were detached, collected by centrifugation, resuspended in SorC buffer, and added to the bacterial suspension to a concentration of 5 x 10^2 amoebal cells per ml. Subsequently, 200 µl of the homogenate was plated on agar plates made of 5-fold diluted SM medium (SM medium according to (Sussman, 1987)). Plates were incubated for 3 to 5 days at 24°C, followed by the enumeration of the number of *D. discoideum* plaques. Three independent experiments were performed, each containing three technical replicates, and a two-tailed Student's *t*-test was performed to ascertain statistical significance.

Hemolysin activity on blood agar plates

The hemolytic activity of *V. cholerae* was assayed using trypticase soy agar containing 5% sheep blood (BD, Allschwil, Switzerland) supplemented with 1 mM EDTA. The respective overnight cultures were spotted onto the plates and incubated at 30°C for 24 h to 30 h. The WT and a *hlyA*-minus mutant served as the positive and negative control, respectively.

Lecithinase / thermolabile hemolysin activity on egg yolk plates

To assess the activity of the lecithinase (also known as thermolabile hemolysin/phospholipase), *V. cholerae* bacteria grown overnight were spotted onto egg yolk-containing agar plates and incubated at 30°C for 72 h. Plates were freshly prepared as follows: a 30% egg yolk (Sigma-Aldrich/Fluka, Buchs, Switzerland) suspension in sterile water was prepared and subsequently diluted 1:20 in autoclaved, warm LB agar before plates were poured. The WT and a *lec*-minus mutant strain served as the positive and negative control, respectively.

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