Supporting Information for

The β-encapsulation cage of rearrangement hotspot (Rhs) effectors is required for type VI secretion

Supporting Information Materials and Methods

Tables S1 to S3

References for SI citations

Figures S1 to S5

Movies S1 to S10

Materials and Methods

Bacterial Strains. Bacterial strains are listed in **Tables S1**. All ECL strains are derivatives *E. cloacae* subsp. *cloacae* ATCC 13047. Bacteria were cultured at 37 °C in shaking lysogeny broth (LB) or on LB-agar supplemented with 150 μg/mL ampicillin (Amp), 66 μg/mL chloramphenicol (Cm), 50 μg/mL kanamycin (Kan), 100 μg/mL spectinomycin (Spc), 200 μg/ml rifampicin (Rif) or 25 μg/mL tetracycline (Tet) where appropriate. For competition co-cultures, inhibitor and target bacteria were grown in LB media supplemented with appropriate antibiotics to mid-log phase, then collected by centrifugation and re-suspended in 1× M9 salts. *E. coli* X90 was used as a target strain for all inter-species competitions. Inhibitor and target cells were mixed at a 1:1 ratio (at OD_{600} ~17), spread onto LB-agar without antibiotics and incubated at 37 °C for 4 h. Aliquots were taken at the beginning and end of co-culture to quantify viable inhibitor- and target-cell colony forming units on antibiotic-selective media. Competitive indices were calculated as the final ratio of inhibitor to target cells divided by the initial ratio.

The *tle* (ECL_01553), *tli* (ECL_01554), ECL_03144, ECL_03145, $eagR_A$ (ECL_01566) and $eagR_B$ (ECL_03141) genes were deleted as described (1, 2). Regions upstream and downstream of *tle* were amplified with primers CH3373/CH3374 and CH3375/CH3376 (respectively) and ligated sequentially to

pKAN to generate pCH11848. The SacI/KpnI restriction fragment from pCH11848 was electroporated into ECL that express the phage λ Red recombinase proteins from plasmid pKOBEG (3). Kanamycin-resistant Δ*tle::kan* (CH11876) transformants were selected on LB-agar supplemented with Kan. A homology region downstream of *tli* was amplified with primers CH3377/CH3378 and ligated via XhoI/KpnI to plasmid pSPM. The resulting plasmid was used as a vector to subclone the SacI/BamHI fragment from pCH11848, generating the combined *tle-tli* deletion construct pCH11939. The SacI/KpnI fragment from the latter plasmid was used to delete *tli* from strain CH11876 to generate CH11895. Regions upstream and downstream of ECL_03144 were amplified with primer pairs CH3391/CH3392 and CH3393/CH3394 and ligated sequentially to pKAN to generate plasmid pCH11871. The SacI/KpnI fragment was recombineered into ECL to generate strain CH11716. A region downstream of ECL_03145 was amplified with primers CH3395/CH3396 and ligated to plasmid pCH11871 using EcoRI/KpnI to generate pCH11872. The SacI/KpnI fragment from pCH11872 was recombineered into strain CH1347 to generate CH11902. Regions upstream and downstream of $eagR_A$ (ECL_01566) were amplified using primers CH2901/2902 and CH3775/CH2904, and the fragments cloned sequentially into pKAN to generate plasmid pCH12318. Regions upstream and downstream of $eagR_B$ (ECL_03141) were amplified using primers CH3471/CH3472 and CH3473/CH3474 (respectively), and the fragments cloned sequentially into pKAN to generate plasmid pCH11921. Linear SacI/KpnI fragments from the plasmids were recombineered into ECL to generate strains CH12688 and CH11906.

Homology fragments for the deletion of *tssM2* (ECL_01813), *hcp1* (ECL_01541), *tae4* (ECL_01542) and *tai4* (ECL_01543) were fused directly to antibiotic-resistance cassettes using overlap extension PCR (OE-PCR) (4). Regions upstream and downstream of *tssM2* were amplified with primer pairs CH2976/CH2977 and CH2978/CH2979. Regions upstream and downstream of *hcp1* were amplified with primer pairs CH2982/CH2981 and CH2980/CH2983. Regions upstream and downstream of *tae4* were amplified with primer pairs CH2964/CH2965 and CH2966/CH2967. A region downstream of *tai4* was amplified with CH2968/CH2969. Kan^R and Spc^R cassettes were amplified from pKAN and pSPM (respectively) using primers CH2952/CH2953. The final OE-PCR products were introduced into the ECL genome using phage λ Red-mediated recombination.

rhsA fragments containing in-frame stop codons at positions 251, 967, 1324 and 1330 were amplified with primer pairs CH3558/CH3559, CH3560/CH3561, CH3316/CH3317 and CH3699/CH3698 (respectively). Each fragment was digested with SacI/BamHI and ligated to plasmid pCH10958 (2). *rhsB* fragments containing in-frame stop codons at positions 207, 858, 1162 and 1278 were amplified with primer pairs CH3232/CH3233, CH3318/CH3319, CH3234/CH3235 and CH3234/CH3831 (respectively). Each fragment was ligated to plasmid pKAN using either SacI/BamHI or SacI/NotI restriction sites. The *rhsB-206* and *rhsB-857* constructs were completed by ligation of the EcoRI/KpnI fragment from plasmid pCH11044. A region downstream of *rhsB* was amplified with primers CH2907/CH2834 and ligated via EcoRI/XhoI to complete the *rhsB-1161* and *rhsB-1277* constructs. The VSV-G epitope sequence was added to *vgrG2* by sequential PCR using primer pairs CH3937/CH3945 and CH3937/CH3946. The resulting product was ligated to pCH11502 (2) using SacI/NotI restriction sites to generate plasmid pCH12883. The Spc^R cassette from pSPM was subcloned into pCH12883 using NotI/EcoRI restriction sites to generate plasmid pCH13002. SacI/KpnI fragments from the *rhs* truncation and *vgrG2-VSV* constructs were introduced into the ECL genome using phage λ Red-mediated recombination.

tssB1 (ECL_01539) was amplified with primers ZR155/ZR306 and ligated to pKAN using SacI/SpeI restriction sites. The coding sequence for super folding GFP was amplified with ZR26/ZR27 and ligated to the construct using SpeI/BamHI sites. Finally, a fragment of *tssC1* (ECL_01540) was amplified with ZR22/ZR23 and ligated using EcoRI/KpnI restriction sites to generate plasmid pCH12509. The linearized construct was introduced into the ECL genome using phage λ Red-mediated recombination. Where appropriate for the generation of multiple chromosomal mutations, Kan^R cassettes were removed using FLP recombinase expressed from plasmid pCP20 (5).

Plasmid constructions. The *tai4* immunity gene was amplified with primers CH2792/CH2793 and ligated to pZS21 using EcoRI/BamHI restriction sites to generate plasmid pCH11686. The *tli* immunity gene was amplified with primers CH3418/CH3419 and ligated to pCH450K using KpnI/XhoI restriction sites to generate plasmid pCH494. For complementation studies, the $eagR_A$ and $eagR_B$ genes were amplified with CH4190/CH2911 and CH2886/CH2887, and ligated to pCH450K using KpnI/XhoI

restriction sites to generate plasmids pCH13568 and pCH12033, respectively. The *vgrG2-VSV* coding sequence was amplified from strain CH14452 using primers CH4452/CH4453, then ligated to pBAD24 (6) using EcoRI/XhoI restriction sites to generate plasmid pCH14397. A NsiI/XhoI fragment containing *araC* and *vgrG2-VSV* was excised from pCH14397 and ligated to pZS21(Cm) to generate plasmid pCH248. The *his*$_6$-*eagR*$_A$ coding sequence was amplified with CH5386/CH2911 and ligated via EcoRI/XhoI to pSCBAD (7) to generate plasmid pCH1000. The *eagR*$_A$-*rhsA*-*rhsI*$_A$ cluster was amplified with CH4400/CH4329 and ligated to pSCBAD and pCH450 via EcoRI/XhoI to generate plasmids pCH1587 and pCH14640, respectively. The *eagR*$_A$-*rhsA-250* fragment was amplified with primers CH4400/CH5387 and ligated to pSCBAD via EcoRI/XhoI to generate plasmid pCH1583. *his*$_6$-*eagR*$_A$-*rhsA*-*rhsI*$_A$ and *his*$_6$-*eagR*$_A$-*rhsA-250* were amplified with CH5386/CH4329 and CH5386/CH5387 and ligated to pCH450 via EcoRI/XhoI to generate plasmids pCH5885 and pCH5881, respectively.

The transmembrane helices of RhsA were deleted in two steps. Plasmid pCH5885 was amplified with primers CH5386/CH5414, and the product ligated to pCH5885 using EcoRI and NcoI restriction sites. A downstream fragment was then amplified with CH5415/CH5416 and ligated to the intermediate construct using SpeI/NcoI to generate plasmid pCH1218. pCH1218 was amplified with CH5386/CH5387 and the product ligated via EcoRI/XhoI to the parent plasmid to generate pCH1220. pCH1218 was also amplified with primers CH4400/CH5414 and the product ligated via EcoRI/NcoI to pCH1587 to generate plasmid pCH1588. The conserved DPxGL motif of RhsA was mutated in two steps. First, a SpeI restriction site (that also introduces an Arg1321Ser substitution) was introduced upstream of the motif by amplification with primers CH4328/CH4631 and ligated to plasmid pCH14640 via KpnI/SbfI. The *rhsA-CT/rhsI* module was the amplified with CH4632/CH3636 (DPxGL) and CH4898/CH3636 (APxGL), and the products ligated via SpeI/XhoI to the intermediate construct to generate plasmids pCH15046 and pCH15105. To append C-terminal FLAG epitopes onto the RhsA variants, wild-type, DPxGL and APxGL constructs were amplified with primers CH4328/CH4629 and the products ligated to pCH5885 using KpnI/SbfI to generate plasmids pCH1594 (wild-type), pCH1595 (DPxGL) and pCH1596 (APxGL).

Polyclonal antisera. The *hcp1* coding sequence was amplified with primers CH3020/CH3022 and ligated to pET21 using NcoI/XhoI restriction sites to generate plasmid pCH11163. The *rhsA* coding sequence for Val82 to Ala467 was amplified with primers CH2960/CH2879 and ligated to pET21 in the same manner to generate pCH11463. Both proteins were over-produced in *E. coli* strain CH2016 (8). Overnight cultures were diluted 1:100 in fresh LB media supplemented with Amp and grown to mid-log phase at 37 °C. Protein production was induced with 1.5 mM IPTG for 90 min. Cells were harvested by centrifugation and the pellets frozen at –80°C. Cells were broken by freeze-thaw in urea lysis buffer [8 M urea, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl]. Hcp1-His_6 and RhsA(1-467)-His_6 were purified by Ni^{2+}-affinity chromatography under denaturing conditions in urea lysis buffer and eluted in urea lysis buffer supplemented with 25 mM EDTA. Proteins were judged to be >95% pure by SDS-PAGE analysis. Purified proteins were dialyzed against water, then lyophilized for injection into New Zealand white rabbits to generate polyclonal antisera (Cocalico Biologicals Inc., Reamstown, PA).

Affinity purifications. Overnight cultures of ECL *vgrG2-VSV* strains were diluted to OD_{600} ~0.05 in LB media supplemented with 0.4% L-arabinose and cultured with shaking at 37 °C. Once in log phase, cells were collected by centrifugation then resuspended in immunoprecipitation buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2% glycerol, 1% Triton X-100]. Cells were broken by two passages through a French press. Unbroken cells and insoluble material were removed by centrifugation at 23,000 ×*g* for 10 min at 4 °C. Anti-VSV-G agarose beads (Sigma-Aldrich) were added to clarified lysates, followed by incubation on a rotisserie for 1 h at 4 °C. After extensive washing with immunoprecipitation buffer, proteins were eluted by boiling in SDS sample-loading buffer.

For immunoprecipitation of over-produced RhsA•VgrG2-VSV complexes from ECL (CH13109), cells were grown to late log phase in LB media supplemented with 0.4% arabinose at 37 °C. Cells were collected by centrifugation and frozen at –80 °C. Frozen cells were resuspended in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl supplemented with 100 µg/mL lysozyme and broken sonication. Clarified lysates incubated with anti-VSV-G agarose on a rotisserie for 1 h at 4 °C. Samples of the lysate and the

unbound fraction were saved for SDS-PAGE and immunoblotting. After extensive washing with immunoprecipitation buffer, proteins were eluted by boiling in SDS sample-loading buffer.

ECL strains (CH13104 through CH13109) carrying plasmid pCH1000 (His_6-$EagR_A$) were grown at 37 °C to mid-log phase and protein production was induced with 0.4% L-arabinose for 1.5 h. Cells were collected by centrifugation and re-suspended in binding buffer [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole] supplemented with 50 µg/mL lysozyme. Cells were broken by sonication and lysates clarified by centrifugation at 23,000 ×*g* for 10 min at 4 °C. Ni^{2+}-nitrilotriacetic acid (NTA) agarose resin (50 to 70 µL) was added to the lysates, followed by incubation on a rotisserie for 1 h at 4 °C. Resins were washed with 7.0 mL of binding buffer, and bound protein eluted with 100 µL of binding buffer supplemented with 50 mM EDTA.

His_6-$EagR_A$•RhsA and VgrG2-VSV were co-produced in *E. coli* X90 cells from two compatible arabinose-inducible plasmid constructs. Cells were grown in LB media (3.0 mL) supplemented with 0.4% L-arabinose for 2 h, then harvested by centrifugation and frozen at –80 °C. Cells were resuspended in 1.8 mL of binding buffer supplemented with 50 µg/mL lysozyme and broken by sonication. Aliquots of the clarified lysates were saved for SDS-PAGE and immunoblot analyses. Ni^{2+}-nitrilotriacetic acid (NTA) agarose resin (50 to 70 µL) was added to the lysates, followed by incubation on a rotisserie for 1 h at 4 °C. Supernatants from the Ni^{2+}-NTA binding reactions were saved for SDS-PAGE and immunoblot analyses. Resins were washed with 7.0 mL of binding buffer, and bound protein eluted with 100 µL of binding buffer supplemented with 50 mM EDTA. *E. coli* cells carrying *his_6-$eagR_A$-rhsA-FLAG* constructs were grown at 37 °C to mid-log phase and protein production was induced with 0.4% L-arabinose for 1.5 h. Cells were collected by centrifugation and processed as described above to isolate protein complexes by Ni^{2+}-NTA affinity chromatography.

Immunoblot analysis. Samples were analyzed by Tris-tricine SDS-PAGE on two-tier 7.5%/15% polyacrylamide gels run at 110 V (constant), or 10% polyacrylamide gels run at 110 V for 1 h. Gels were soaked for 10 min in 25 mM Tris, 192 mM glycine (pH 8.6), 10% methanol, then electroblotted to low-fluorescence PVDF membranes using a semi-dry transfer apparatus at 17 V (constant) for 1 h.

Membranes were blocked with 4% non-fat milk in 1× PBS for 30 min at room temperature, and incubated with primary antibodies in 0.1% non-fat milk in 1× PBS overnight at 4 °C. Rabbit polyclonal antisera to Hcp1-His_6 was used at a 1:10,000 dilution and antisera to RhsA(V82-A467)-His_6 was used at a 1:5,000 dilution. Mouse anti-VSV-G (Sigma-Aldrich) was used at a 1:150,000 dilution. Blots were incubated with 800CW-conjugated goat anti-rabbit IgG (1:125,000 dilution, LICOR) or 680LT-conjugated goat anti-mouse IgG (1:125,000 dilution, LICOR) in 1× PBS. Immunoblots were visualized with a LI-COR Odyssey infrared imager.

Hcp1 secretion. ECL cells were grown to mid-log phase in LB media at 37 °C, then collected by centrifugation at 3,000 ×*g* for 5 min. Cell pellets were washed once with 1× M9 salts, then resuspended in urea lysis buffer and subjected to a freeze-thaw cycle to extract proteins for SDS-PAGE and immunoblotting. Culture supernatants were centrifuged again to remove cellular contamination. Supernatants were adjusted to 75% ethanol and incubated overnight at –80 °C. Proteins were precipitated by centrifugation at 21,000 ×*g* for 15 min at 4 °C. Precipitates were washed once with 75% ice-cold ethanol, then air-dried and dissolved in urea lysis buffer. Samples were analyzed by SDS-PAGE using Tris-tricine 10% polyacrylamide gels, which were immunoblotted as described above.

Fluorescence microscopy. ECL strains expressing chromosomal *tssB-gfp* fusions were inoculated from a single colony into 0.5× LB media and cultured with shaking at 37 °C. Once in mid-log phase, cells were collected by centrifugation, then spotted onto a thin pad of 1% agarose in 1× M9 minimal media and covered with a glass coverslip. Time-lapse GFP-fluorescence microscopy was performed with images taken every 5 seconds for 2.5 min per field of view, followed by a bright-field image once the time-lapse was completed. ImageJ was used for all image analysis and manipulations.

Table S1. Bacterial strains

Strain	Description[a]	Reference
X90	*E. coli* F´ *lacI*^{q} *lac´ pro´/ara* Δ*(lac-pro) nal1 argE(amb) rif*^{r} *thi-1*, Rif^{R}	(9)
ECL	*Enterobacter cloacae* subsp. cloacae ATCC 13047, Amp^{R}	ATCC
CH1347	ECL Δ*03144*, Amp^{R}	This study
CH2016	X90 (DE3) Δ*rna* Δ*slyD::kan*, Rif^{R} Kan^{R}	(8)
CH8163	ECL, Amp^{R} Rif^{R}	(2)
CH11178	ECL Δ*rhsA::kan*, Amp^{R} Kan^{R}	(2)
CH11179	ECL Δ*rhsA*, Amp^{R}	(2)
CH11181	ECL Δ*rhsA* Δ*rhsI*$_{A}$*::kan*, Amp^{R} Kan^{R}	(2)
CH11185	ECL Δ*rhsA* Δ*rhsI*$_{A}$, Amp^{R} Rif^{R}	(2)
CH11186	ECL Δ*rhsB::kan*, Amp^{R} Kan^{R}	(2)
CH11188	ECL Δ*rhsB* Δ*rhsI*$_{B}$*::spc*, Amp^{R} Spc^{R}	(2)
CH11196	ECL Δ*tssM1::kan*, Amp^{R} Kan^{R}	(2)
CH11199	ECL Δ*hcp1::spc*, Amp^{R} Spc^{R}	This study
CH11202	ECL Δ*tae4::spc*, Amp^{R} Spc^{R}	This study
CH11204	ECL Δ*tae4* Δ*tai4::kan*, Amp^{R} Kan^{R}	This study
CH11205	ECL Δ*tae4* Δ*tai4*, Amp^{R}	This study
CH11223	ECL Δ*rhsB* Δ*rhsI*$_{B}$*::spc*, Amp^{R} Spc^{R} Rif^{R}	(2)
CH11396	ECL Δ*tssM1*, Amp^{R}	This study
CH11436	ECL Δ*vgrG2::kan*, Amp^{R} Kan^{R}	(2)
CH11530	ECL Δ*rhsA rhsB(L207amb)::kan*, Amp^{R} Kan^{R}	This study
CH11531	ECL Δ*rhsA rhsB(E1162amb)::kan*, Amp^{R} Kan^{R}	This study
CH11642	ECL Δ*rhsA rhsB(D858amb)::kan*, Amp^{R} Kan^{R}	This study
CH11716	ECL Δ*03144::kan*, Amp^{R} Kan^{R}	This study
CH11733	ECL Δ*rhsB*, Amp^{R}	This study
CH11748	ECL Δ*rhsB* Δ*rhsA::kan*, Amp^{R} Kan^{R}	This study
CH11749	ECL Δ*rhsB rhsA(1323)::kan*, Amp^{R} Kan^{R}	This study
CH11876	ECL Δ*tle::kan*, Amp^{R} Kan^{R}	This study
CH11895	ECL Δ*tle* Δ*tli::spc*, Amp^{R} Spc^{R}	This study
CH11902	ECL Δ*03144* Δ*03145::kan*, Amp^{R} Kan^{R}	This study
CH11903	ECL Δ*rhsB* Δ*rhsA*, Amp^{R}	This study

CH11906	ECL Δ*eagR*$_B$*::kan*, Amp^R Kan^R	This study
CH12037	ECL Δ*tssM2::kan*, Amp^R Kan^R	This study
CH12038	ECL Δ*tssM1* Δ*tssM2::kan*, Amp^R Kan^R	This study
CH12226	ECL Δ*rhsB rhsA(966)::kan*, Amp^R Kan^R	This study
CH12384	ECL Δ*vgrG1::kan*, Amp^R Kan^R	(2)
CH12385	ECL Δ*rhsB* Δ*vgrG1::kan*, Amp^R Kan^R	This study
CH12386	ECL Δ*rhsB* Δ*vgrG2::kan*, Amp^R Kan^R	This study
CH12391	ECL Δ*vgrG1*, Amp^R	(2)
CH12414	ECL Δ*vgrG1* Δ*vgrG2::kan*, Amp^R Kan^R	(2)
CH12415	ECL Δ*rhsA* Δ*vgrG1::kan*, Amp^R Kan^R	This study
CH12416	ECL Δ*rhsA* Δ*vgrG2::kan*, Amp^R Kan^R	This study
CH12482	ECL *tssB1-sfGFP::kan*, Amp^R Kan^R	This study
CH12483	ECL *tssB1-sfGFP*, Amp^R	This study
CH12485	ECL *tssB1-sfGFP* Δ*rhsA::kan*, Amp^R Kan^R	This study
CH12486	ECL Δ*rhsA rhsB(Q1278och)::kan*, Amp^R Kan^R	This study
CH12492	ECL Δ*rhsB rhsA(1329)::kan*, Amp^R Kan^R	This study
CH12497	ECL Δ*rhsB rhsA(250)::kan*, Amp^R Kan^R	This study
CH12555	ECL *tssB1-sfGFP* Δ*rhsA*, Amp^R	This study
CH12561	ECL *tssB1-sfGFP* Δ*tssM1::kan*, Amp^R Kan^R	This study
CH12562	ECL *tssB1-sfGFP* Δ*vgrG1::spc*, Amp^R Spc^R	This study
CH12564	ECL *tssB1-sfGFP* Δ*vgrG1::spc* Δ*vgrG2::kan*, Amp^R Spc^R Kan^R	This study
CH12582	ECL Δ*vgrG2*, Amp^R	This study
CH12594	ECL *tssB1-sfGFP* Δ*rhsA* Δ*rhsB::kan*, Amp^R Kan^R	This study
CH12609	ECL *tssB1-sfGFP* Δ*rhsB*, Amp^R	This study
CH12612	ECL *tssB1-sfGFP* Δ*rhsB rhsA(250)::kan*, Amp^R Kan^R	This study
CH12613	ECL *tssB1-sfGFP* Δ*rhsB rhsA(966)::kan*, Amp^R Kan^R	This study
CH12614	ECL *tssB1-sfGFP* Δ*rhsB rhsA(1323)::kan*, Amp^R Kan^R	This study
CH12615	ECL *tssB1-sfGFP* Δ*rhsB rhsA(1329)::kan*, Amp^R Kan^R	This study
CH12688	ECL Δ*eagR*$_A$*::kan*, Amp^R Kan^R	This study
CH13104	ECL Δ*rhsB::kan vgrG2-VSV::spc*, Amp^R Kan^R Spc^R	This study
CH13105	ECL Δ*rhsB rhsA(250)::kan vgrG2-VSV::spc*, Amp^R Kan^R Spc^R	This study

CH13106	ECL Δ*rhsB rhsA(966)::kan vgrG2-VSV::spc*, Amp^R Kan^R Spc^R	This study
CH13107	ECL Δ*rhsB rhsA(1323)::kan vgrG2-VSV::spc*, Amp^R Kan^R Spc^R	This study
CH13108	ECL Δ*rhsB rhsA(1329)::kan vgrG2-VSV::spc*, Amp^R Kan^R Spc^R	This study
CH13109	ECL Δ*rhsB* Δ*rhsA::kan vgrG2-VSV::spc*, Amp^R Kan^R Spc^R	This study
CH13230	ECL Δ*tssM1::kan vgrG2-VSV::spc*, Amp^R Kan^R Spc^R	This study
CH13286	ECL Δ*rhsA::kan vgrG2-VSV::spc*, Amp^R Kan^R Spc^R	This study
CH15044	ECL Δ*rhsA* Δ*tae4::spc*, Amp^R Spc^R	This study
CH15045	ECL Δ*rhsB* Δ*tae4::spc*, Amp^R Spc^R	This study

[a]Abbreviations: Amp^R, ampicillin-resistant; Kan^R, kanamycin-resistant; Rif^R, rifampicin-resistant; Spc^R, Spectinomycin-resistant

Table S2. Plasmids

Plasmid number	**Description[a]**	**Reference**
pBAD24	Carries *araC* and P_{BAD} promoter, Amp^R	(6)
pCP20	temperature sensitive replication origin, expresses FLP recombinase. Amp^R, Cm^R	(5)
pKOBEG	temperature sensitive replication origin, expresses the Bet-Gam-Exo proteins from phage λ. Cm^R	(3)
pSCBAD	Carries *araC* and P_{BAD} promoter, Tp^R	(7)
pCH70	pKAN, Kan^R, Amp^R	(10)
pCH248	pZSBAD::*vgrG2-VSV*, Cm^R	This study
pCH450	pACYC184 derivative that carries *araC* and P_{BAD} promoter, Tet^R	(8)
pCH450K	derivative of pCH450 with 5´-Kpn cloning site, Tet^R	(2)
pCH494	pCH450K::*tli*, Tet^R	This study
pCH1000	pSCBAD::*his$_6$-eagR$_A$*, Tp^R	This study
pCH1218	pCH450::*his$_6$-eagR$_A$-rhsA(ΔTM)-rhsI*, Tet^R	This study
pCH1220	pCH450::*his$_6$-eagR$_A$-rhsA(ΔTM)-250*, Tet^R	This study
pCH1583	pSCBAD::*eagR$_A$-rhsA-250*, Tp^R	This study
pCH1587	pSCBAD::*eagR$_A$-rhsA-rhsI$_A$*, Tp^R	This study
pCH1588	pSCBAD::*eagR$_A$-rhsA(ΔTM)-rhsI$_A$*, Tp^R	This study
pCH1594	pCH450::*his$_6$-eagR$_A$-rhsA-FLAG*, Tet^R	This study
pCH1595	pCH450::*his$_6$-eagR$_A$-rhsA(R1321S)-FLAG*, Tet^R	This study
pCH1596	pCH450::*his$_6$-eagR$_A$-rhsA(R1321S/D1323A)-FLAG*, Tet^R	This study

pCH5881	pCH450::*his*$_6$-*eagR*$_A$-*rhsA-250*, Tet^R	This study
pCH5885	pCH450::*his*$_6$-*eagR*$_A$-*rhsA-rhsI*$_A$, Tet^R	This study
pCH9384	pSPM, Spc^R, Amp^R	(11)
pCH10958	pKAN::Δ*rhsA*, Kan^R, Amp^R	(2)
pCH11044	pKAN::Δ*rhsB*, Kan^R, Amp^R	(2)
pCH11050	pKAN::Δ*tssM1*, Kan^R, Amp^R	(2)
pCH11163	pET21P::*hcp1*, Amp^R	This study
pCH11430	pTrc99-Cm, Cm^R	(2)
pCH11459	pSPM::Δ*vgrG1*, Spc^R, Amp^R	(2)
pCH11463	pET21P::*rhsA(82-467)*, Amp^R	This study
pCH11502	pKAN::Δ*vgrG2*, Kan^R, Amp^R	(2)
pCH11632	pKAN::*rhsB(L207amb)*, Kan^R, Amp^R	This study
pCH11633	pKAN::*rhsB(E1162amb)*, Kan^R, Amp^R	This study
pCH11635	pKAN::*rhsB(D858amb),* Kan^R, Amp^R	This study
pCH11636	pKAN::*rhsA(D1324amb)*, Kan^R, Amp^R	This study
pCH11686	pZS21::*tai4*, Kan^R	This study
pCH11138	pTrc99-Cm::*rhsI*$_B$, Cm^R	(2)
pCH11848	pKAN::Δ*tle*, Amp^R Kan^R	This study
pCH11871	pKAN::Δ*03144*, Amp^R Kan^R	This study
pCH11872	pKAN::Δ*03145*, Amp^R Kan^R	This study
pCH11921	pKAN::Δ*eagR*$_B$, Kan^R, Amp^R	This study
pCH11981	pKAN::*rhsA(M251och)*, Kan^R, Amp^R	This study
pCH11939	pSPM::Δ*tle-tli*, Amp^R Spc^R	This study
pCH12003	pKAN::*rhsA(H967och)*, Kan^R, Amp^R	This study
pCH12033	pCH450K::*eagR*$_B$, Tet^R	This study
pCH12291	pKAN::*rhsA(Q1330och)*, Kan^R, Amp^R	This study
pCH12318	pKAN::Δ*eagR*$_A$, Kan^R, Amp^R	This study
pCH12370	pKAN::Δ*vgrG1*, Kan^R, Amp^R	(2)
pCH12437	pKAN::*rhsB(Q1278och)*, Kan^R, Amp^R	This study
pCH12509	pKAN::*tssB1-sfGFP*, Kan^R, Amp^R	This study
pCH12799	pZS21::*rhsI*$_A$, Kan^R	(2)

pCH12883	pKAN::*vgrG2-VSV*, Kan^R, Amp^R	This study
pCH13002	pSPM::*vgrG2-VSV*, Spc^R, Amp^R	This study
pCH13568	pCH450K::$eagR_A$, Tet^R	This study
pCH14397	pBAD24::*vgrG2-VSV*, Amp^R	This study
pCH14640	pCH450::$eagR_A$-*rhsA*-$rhsI_A$, Tet^R	This study
pCH15046	pCH450::$eagR_A$-*rhsA(R1321S)*-$rhsI_A$, Tet^R	This study
pCH15105	pCH450::$eagR_A$-*rhsA(R1321S/D1323A)*-$rhsI_A$, Tet^R	This study

[a]Abbreviations: Amp^R, ampicillin-resistant; Cm^R, chloramphenicol-resistant; Kan^R, kanamycin-resistant; Tet^R, tetracycline-resistant; Tp^R, trimethoprim-resistant; Spc^R, spectinomycin-resistant

Table S3. Oligonucleotides

Identifier	Description[a]	Sequence[b]	Reference
CH2792	tai4-Eco-for	5´ - TTT GAA TTC TTC TGG AGC CTG AAA TGA AAA AG	This study
CH2793	tai4-Bam-rev	5´ - TTT GGA TCC CTA CTT TGA GGA TTT GAG TGG	This study
CH2834	$rhsI_B$-Xho-rev	5´ - TTT CTC GAG GTA TCC TAG CCA TAA AAA TAA TC	(2)
CH2879	rhsA-A467-Spe-rev	5´ - AAA ACT AGT GGC AGC GGT TAC GCG CTG TGG	This study
CH2886	$eagR_B$-Kpn-for	5´ - AAT GGT ACC ATG ATC GCT TTT CCT GAG GG	This study
CH2887	$eagR_B$-Xho-rev	5´ - ATC CCT CGA GTT AAT GGT GGA AGC G	This study
CH2901	$eagR_A$-KO-Sac	5'- TTT GAG CTC ATG CTC CGC TGC GTT ATA	This study
CH2902	$eagR_A$-KO-Spe	5´ - TTT ACT AGT CGT GTT ATC CTG CCA GGC	This study
CH2904	$eagR_A$-KO-Kpn	5´ - TTT GGT ACC CAG AGA GCA ACA TGC CGG	This study
CH2907	rhsB-KO-Eco	5´ - TTT GAA TTC CAA TGA ATA TGC TGA ATG TGA G	(2)
CH2911	$eagR_A$-Xho-rev	5´ - TCA CTC GAG CCT TAC ACA TTC CGG	This study
CH2952	pKAN-OE-for	5´ - CCG CTC TAG AAC TAG TGG	This study
CH2953	pKAN-OE-rev	5´ - GTC GAC GGT ATC GAT AAG C	This study
CH2960	rhsA-V82-Nco-for	5´ - TGG CCA TGG TTA CTG ACG ATA TCA G	This study
CH2964	tae4(KO)-Sac	5´ - GGG GAG CTC CCC AGC CAG GTA ATA TG	This study
CH2965	tae4(KO)-OE-rev	5´ - CCA CTA GTT CTA GAG CGG CTT GTT TCT CCT TGA AAA G	This study
CH2966	tae4(KO)-OE-for	5´ - GCT TAT CGA TAC CGT CGA CAC CTT CTG GAG CCT GAA ATG	This study

CH2967	tae4(KO)-Kpn	5′ - TCT GAT AAT GAC CAG GCT CGG TAC C	This study
CH2968	tai4(KO)-OE-for	5′ - GCT TAT CGA TAC CGT CGA CTA GTA AAG ATG AAA TCG GC	This study
CH2969	tai4(KO)-Kpn	5′ - ATA GGT ACC GTC ACT TCG ATG CGG	This study
CH2976	tssM2-KO-Sac	5′ - TTT GAG CTC GGC AAC CGC CTG ACA C	This study
CH2977	tssM2(KO)-OE-rev	5′ - CCA CTA GTT CTA GAG CGG CTT CCG TAG TCT TCG GTG C	This study
CH2978	tssM2 (KO)-OE-for	5′ - GCT TAT CGA TAC CGT CGA CGG ACA GTA CGG AAA GCA G	This study
CH2979	tssM2(KO)-Kpn	5′ - TTT GGT ACC GCC GAG CCA TTC	This study
CH2980	hcp1(KO)-OE-for	5′ - GCT TAT CGA TAC CGT CGA CGT AGT GGG TCC GAA AGG G	This study
CH2981	hcp1(KO)-OE-rev	5′ - CCA CTA GTT CTA GAG CGG CTA CTC TTC GTC GAT GAA C	This study
CH2982	hcp1(KO)-Sac	5′ - TTT GAG CTC CCA GGT GCA GGA GAT TC	This study
CH2983	hcp1(KO)-Kpn	5′ - AAA GGT ACC TTC CAG AGT GTT ACA TGC	This study
CH3020	hcp1-Nco-for	5′ - ATA CCA TGG CTA TTG ATA TGT TTC	This study
CH3022	hcp1-Xho-rev	5′ - CTA CTC GAG TGC TTC TTT GTT TTC TTT G	This study
CH3232	rhsB-G47-Sac-for	5′ - GCC GAG CTC GGC GCA TCC TGC CTT GGC	This study
CH3233	rhsB-L207amb-Bam-rev	5′ - CAG GGA TCC TGC CCA GCT ACT TAG AGA GCG C	This study
CH3234	rhsB-E1001-Sac-for	5′ - TAC GAG CTC GAA GGG CGT CTG CTG AAG C	This study
CH3235	rhsB-E1162amb-Bam-rev	5′ - TGT GGA TCC AGT AAA TCT AAC CGC TGC TCT GG	This study
CH3316	rhsA-T1133-Sac-for	5′ - TTT GAG CTC ACA GAA GTG ATC AGC CAG	This study
CH3317	rhsA-D1324amb-Bam-rev	5′ - TTT GGA TCC CTA TAT TCG GGT TAG ACT ATT AGC	This study
CH3318	rhsB-L683-Sac-for	5′ - TTT GAG CTC TGC TGA GTG CCG TGA TC	This study
CH3319	rhsB-D858amb-Bam-rev	5′ - TTT GGA TCC ACT AGC TTT CGA TAC CCA GCG C	This study
CH3373	tle(KO)-Sac	5′ - CAA GAG CTC CGG GAT GGT TGC C	This study
CH3374	tle(KO)-Bam	5′ - ATT GGA TCC GTC CTG TTA CCA GTC	This study
CH3375	tle(KO)-Xho	5′ - AGG CTC GAG ACA TTT CAA TTA TTA GG	This study
CH3376	tle(KO)-Kpn	5′ - AAC GGT ACC TGG CGA TAA ACC CGC	This study
CH3377	tli(KO)-Xho	5′ - CCA ACT CGA GTT AAA TAG GAA ACG	This study

CH3378	tli(KO)-Kpn	5′ - CCA GGT ACC AAA GTG CTG TGT GC	This study
CH3391	3144(KO)-Sac	5′ - GGT GAG CTC CGC ATA TGT GTT TAA GG	This study
CH3392	3144(KO)-Bam	5′ - CAT GGA TCC CTC TAC TTT ATA TGG	This study
CH3393	3144(KO)-Eco	5′ - AGA GAA TTC ATG AGG TTG TTA AAT AA	This study
CH3394	3144(KO)-Kpn	5′ - TCC GGT ACC TTT GCT TAA AGG G	This study
CH3395	3145(KO)-Eco	5′ - CAA GAA TTC AGG AAA AAA TTG ATT TTA	This study
CH3396	3145(KO)-Kpn	5′ - TTA GGT ACC TCG ATC CTT GCC G	This study
CH3418	tli-Kpn-for	5′ - GAG GGT ACC ATG AAA TCG TTC TTA TCA GGC	This study
CH3419	tli-Xho-rev	5′ - ATA CTC GAG CTA TTT AAC CGG AGT TGG TG	This study
CH3471	eagR$_B$-KO-Sac	5′ - AAT GAG CTC ACT TTA TAT GGA AAT AAT CC	This study
CH3472	eagR$_B$-KO-Bam	5′ - AAA GGA TCC CAT TCC TTC ATT AAA CAG GCA C	This study
CH3473	eagR$_B$-KO-Eco	5′ - CTT GAA TTC CCA CCA TTA ACA CCA GGG	This study
CH3474	eagR$_B$-KO-Kpn	5′ - ATC GGT ACC GAT GAA GAC GTT GTC CGA G	This study
CH3558	rhsA-N78-Sac-for	5′ - GCG GAG CTC CTA ACC TGG CGG GTG	This study
CH3559	rhsA-M251och-Bam-rev	5′ - GAG GGA TCC TTA CCC CAG TGC CAG C	This study
CH3560	rhsA-R801-Sac-for	5′ - GGT GAG CTC CCG CTG GGA CAG C	This study
CH3561	rhsA-H967och-Bam-rev	5′ - GCT GGA TCC TTA CCC GCT GCC GTA G	This study
CH3636	rhsI$_A$-Xho-rev	5′ - GTG CTC GAG CAT TAA CAT ATT AAA TCG	This study
CH3698	rhsA-Q1330och-Bam-rev	5′ - AAC GGA TCC TTA TTT TAA TCC CAG AGG GTC	This study
CH3699	rhsA-R1041-Sac-for	5′ - TAA GAG CTC GCG GGA AGA ACG GG	This study
CH3775	eagR$_A$(KO)-Eco	5′ - GAT GAA TTC TAC TCT CTC GGC ACT CAG	This study
CH3831	rhsB-Q1278och-Not-rev	5′ - AAC GCG GCC GCT TAC GCG GAG AGT CCC CAC	This study
CH3914	rhsA-Eco-for	5′ - TTT GAA TTC GGC ATG AGC GAT AAC AAC	This study
CH3937	vgrG2-T401-Sac	5′ - ACA CGA GCT CCT GCT GGG TG	This study
CH3938	rhsA(1330)-Xho-rev	5′ - TTT CTC GAG CTT ATT TTA ATC CCA GAG GGT CTA TTC	This study
CH3945	vgrG2-VSV-rev	5′ - CAA GAC GAT TCA TTT CAA TAT CAG TAT AAC TAG TAT CAC CCT TGG TCG TGA ATT TCG C	This study
CH3946	VSV-Not-rev	5′ - TTT GCG GCC GCA TCC TTA TTT GCC AAG ACG ATT CAT TTC AAT ATC AGT AT	This study

CH4190	eagR_A-Kpn-for	5′- AGT GGT ACC ATG AAA TAC ACC CTC CAG G	This study
CH4328	rhsA-W1051-Kpn-for	5′ - TTC AGC TGG TAC CGC TGG	This study
CH4329	rhsI_A-Xho-rev	5′ - ACC CTC GAG CCT GCA GGT GTG GTC GAA CAT TAA CAT ATT AAA TCG	This study
CH4400	eagR_A-Eco-for	5′ - AGT GAA TTC ATG AAA TAC ACC CTC CAG G	This study
CH4452	vgrG2-Eco-for	5′ - ATA GAA TTC ATG CTC AAC CGA ATT ACC	This study
CH4453	VSV-Xho-rev	5′ - TGC CTC GAG ATC CTT ATT TGC CAA GAC G	This study
CH4629	rhsA-FLAG-Xho/Sbf-rev	5′ - GGT CCT GCA GGC TCG AGT TAT TTG TCA TCA TCG TCC TTG TAG TCA GTA ATC CTC TTG CCA	This study
CH4631	rhsA-R1321S-Spe-Xho-Sbf-rev	5′ - GGT CCT GCA GGC TCG AGG GTC TAT ACT AGT TAG ACT ATT AGC ACC	This study
CH4632	rhsA-R1321S-Spe-for	5′ - AGT CTA ACT AGT ATA GAC CCT CTG GG	This study
CH4898	rhsA-D1323A-Spe-for	5′ - CTA ACT AGT ATA GCC CCT CTG GGA TTA AAA C	This study
CH5386	eagR_A-His_6-Eco-for	5′ - GAG GAA TTC ATG CAC CAT CAT CAT CAT CAT CAT TCT ATG AAA TAC ACC CTC CAG GAA GGT TCG	This study
CH5387	rhsA(250)-Xho-rev	5′ - TTT CTC GAG TTA CCC CAG TGC CAG CCC	This study
CH5388	rhsA(966)-Xho-rev	5′ - TTT CTC GAG TTA CCC GCT GCC GTA GTA GAA C	This study
CH5389	rhsA(1323)-Xho-rev	5′ - TTT CTC GAG TTA TAT TCG GGT TAG ACT ATT AGC ACC	This study
CH5414	rhsA(T23)-Nco/Spe-rev	5′ - CCG CCA TGG TAC TAG TGA TAT CCG CGA AAA TGC	This study
CH5415	rhsA(A80)-Spe-for	5′ - ATC ACT AGT GCG GGT GTT ACT GAC GAT ATC AG	This study
CH5416	rhsA-Nco-rev	5′ - AGG CCA TGG TCG TTA TAG CGG	This study
ZR22	tssB(KO)-Eco	5′ - TTT GAA TTC AAA GGT AGC GAG GAG TAA TCG	This study
ZR23	tssB(KO)-Kpn	5′ - TTT GGT ACC GCT GTG GTC AAA GTA GTA GTC G	This study
ZR26	sfGFP-Spe-for	5′ - TTT ACT AGT GCA GCA GCT GGT GGA GGT AGC AAA GGA GAA GAA CTT TTC AC	This study
ZR27	sfGFP-Bam-rev	5′ - TTT CTG CAG GAT CCT TAT TTG TAG AGC TCA TCC ATG CC	This study
ZR155	tssB-Sac-for	5′ - TTT GAG CTC GCA GTT GCT GAA AAC CGT G	This study
ZR306	tssB-Spe-rev	5′ - TTT ACT AGT CTC CTC GCT ACC TTT CGC ACT TTC GTC ATT TTT CGG CAA CTG GCT	This study

[b]Restriction endonuclease sites are underlined.

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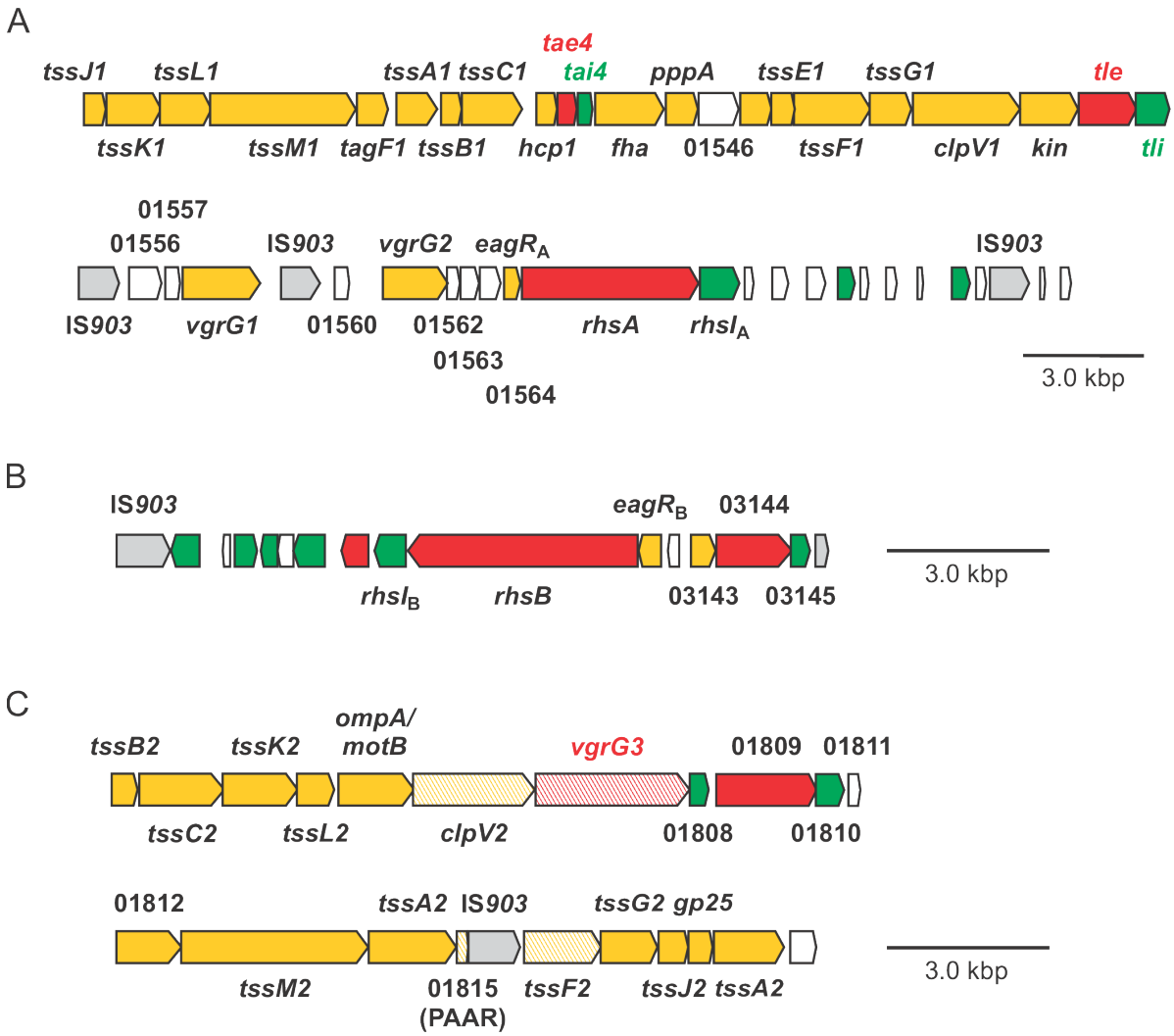

Figure S1. ECL T6SS loci. A) The T6SS-1 locus of *E. cloacae* ATCC 13047. Gene encoding hypothetical proteins of unknown function are indicated by their corresponding ordered locus number. B) The *eagR*$_B$-*rhsBI* locus of *E. cloacae* ATCC 13047. C) The T6SS-2 locus of *E. cloacae* ATCC 13047. Hatched fills indicate pseudogenes as described in the manuscript text. For all panels, effector genes are rendered in red and predicted immunity genes in green. IS*903* elements are shown in grey.

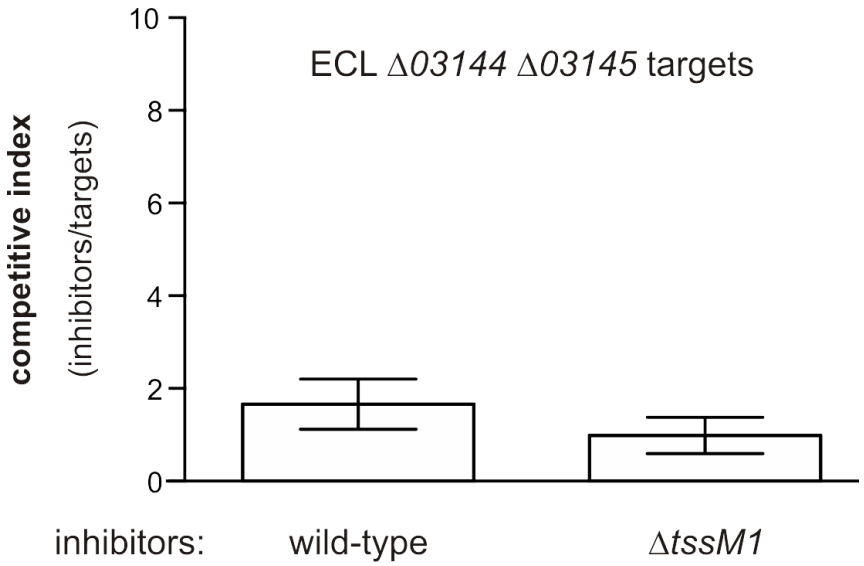

Figure S2. Analysis of a predicted PAAR-domain containing T6SS-1 effector. ECL Δ*03144* Δ*03145* target bacteria were co-cultured at a 1:1 ratio with wild-type of Δ*tssM1* ECL inhibitor strains for 4 h. The competitive index is the ratio of inhibitor to target bacteria at 4 h divided by the initial ratio. Data are the average ± SEM for three independent experiments.

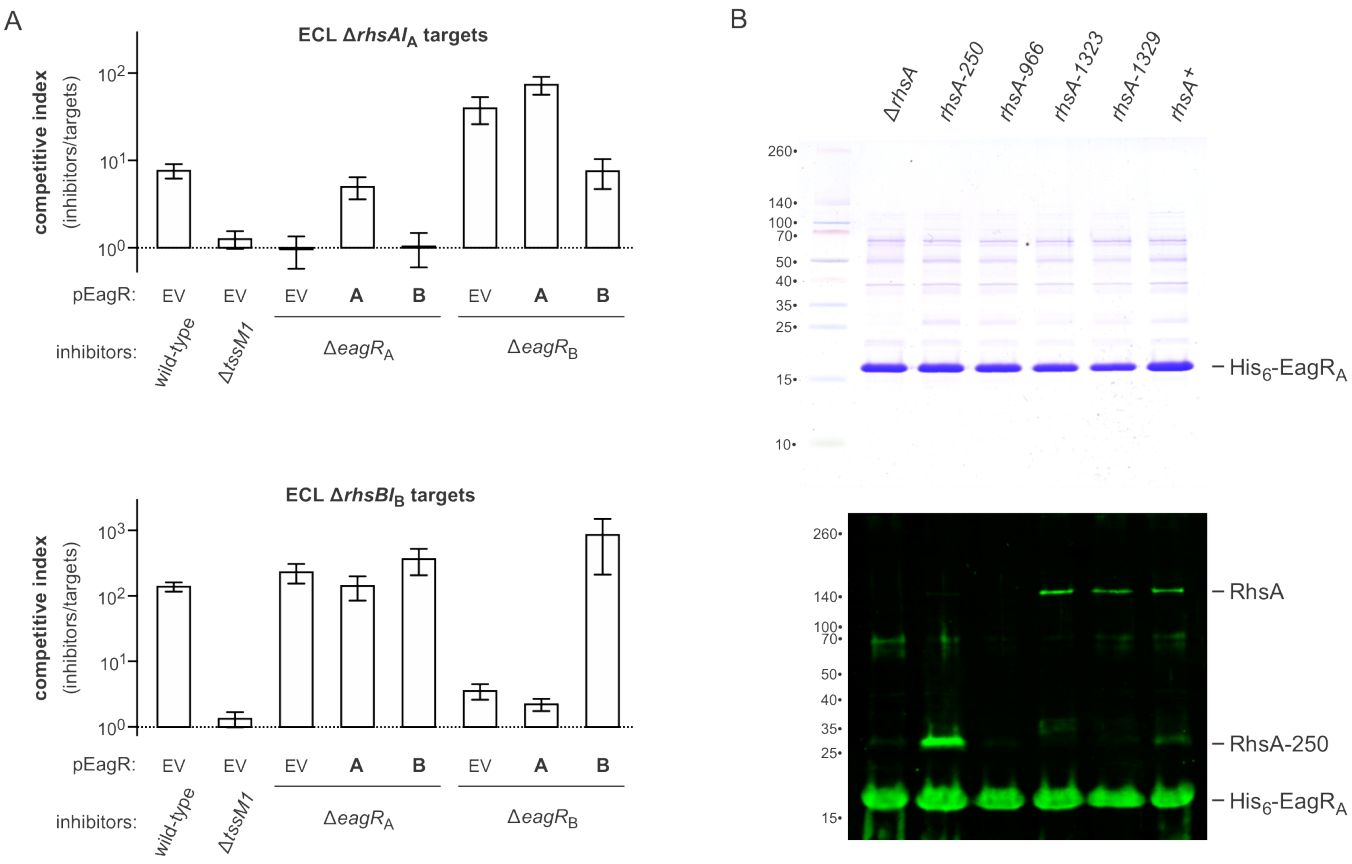

Figure S3. EagR proteins are required for Rhs effector activity. A) Target bacteria were co-cultured at a 1:1 ratio with ECL inhibitor strains for 4 h. Where indicated, the inhibitor strains were complemented with plasmid-borne *eagR*$_A$, *eagR*$_B$ or empty vector (EV). The competitive index is the ratio of inhibitor to target bacteria at 4 h divided by the initial ratio. Data are the average ± SEM for three independent experiments. B) Truncated RhsA proteins accumulate in ECL. Over-produced His$_6$-EagR$_A$ was isolated by Ni^{2+}-affinity chromatography from ECL Δ*rhsB* strains that harbor the indicated *rhsA* alleles. Samples were analyzed by SDS-PAGE (top) and immunoblotting with polyclonal antibodies to RhsA (bottom). Because His$_6$-tagged RhsA was used as an antigen, the antisera cross-reacts with the His$_6$ epitope on EagR$_A$.

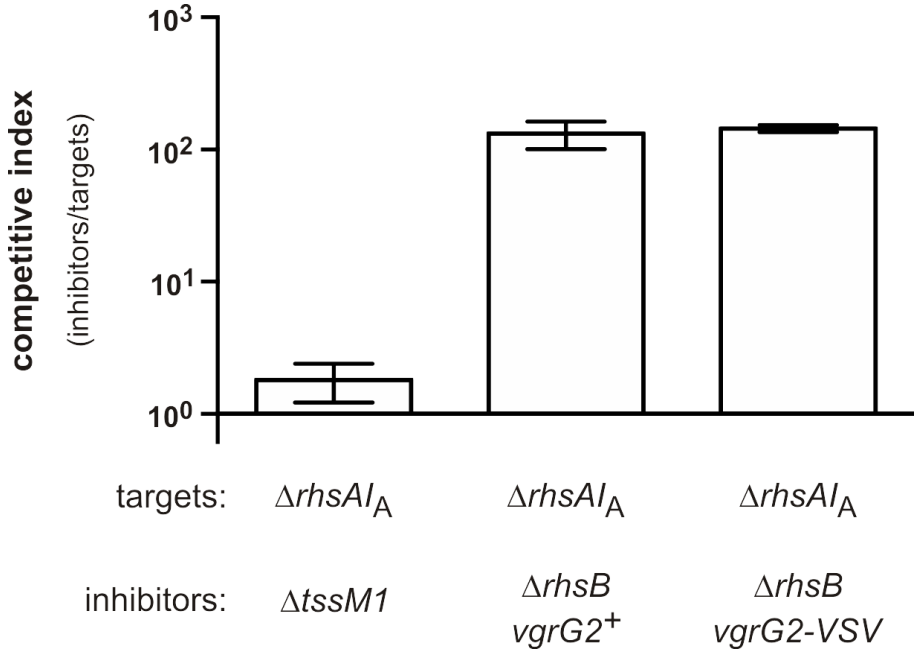

Figure S4. VgrG2-VSV supports T6SS-1 activity. ECL Δ*rhsA* Δ*rhsI*$_A$ target bacteria were co-cultured at a 1:1 ratio with the indicated ECL inhibitor strains for 4 h. The competitive index is the ratio of inhibitor to target bacteria at 4 h divided by the initial ratio. Data are the average ± SEM for three independent experiments.

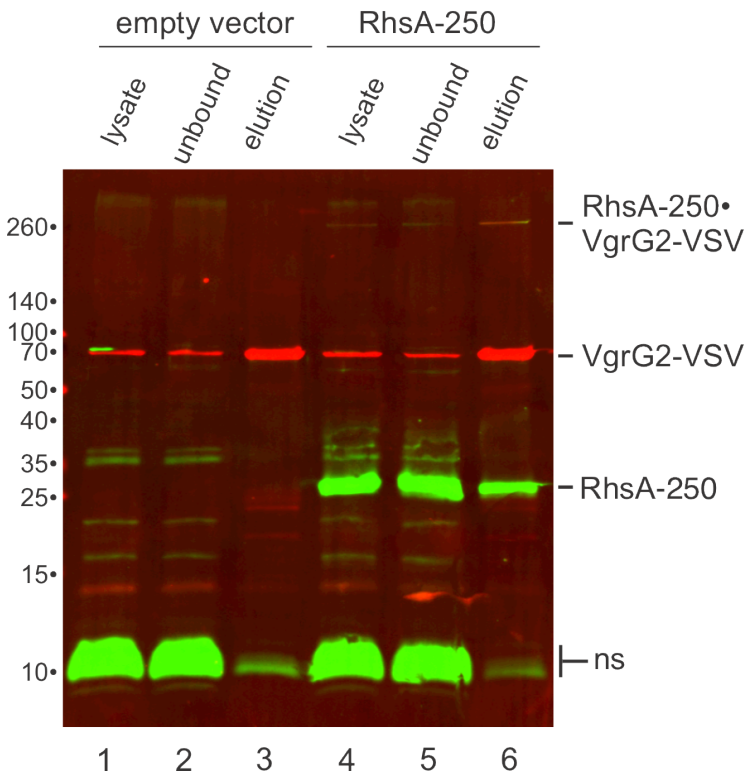

Figure S5. Over-produced RhsA-250 stabilizes VgrG2-VSV trimers in ECL. $EagR_A$ and RhsA-250 were over-produced with VgrG2-VSV in ECL Δ*rhsB* Δ*rhsA* cells for isolation by anti-VSV immunoprecipitation. Crude lysates and the unbound and elution fractions from anti-VSV agarose resin were resolved on two-layer 7.5%/15% polyacrylamide gels and immunoblotted with antibodies to RhsA (green) and VSV-G (red). Non-specific (ns) ECL proteins detected by RhsA antisera are indicated.

Supporting Information Movies

Movie S1. Time-lapse microscopy of ECL *tssB1-gfp* cells (CH12483).

Movie S2. Time-lapse microscopy of ECL *tssB1-gfp* Δ*tssM1* cells (CH12561).

Movie S3. Time-lapse microscopy of ECL *tssB1-gfp* Δ*rhsA* Δ*rhsB* cells (CH12594).

Movie S4. Time-lapse microscopy of ECL *tssB1-gfp* Δ*vgrG1* Δ*vgrG2* cells (CH12564).

Movie S5. Time-lapse microscopy of ECL *tssB1-gfp* Δ*rhsB rhsA*$^{+}$ cells (CH12609).

Movie S6. Time-lapse microscopy of ECL *tssB1-gfp* Δ*rhsB rhsA-1323* cells (CH12614).

Movie S7. Time-lapse microscopy of ECL *tssB1-gfp* Δ*rhsB rhsA-1330* cells (CH12615).

Movie S8. Time-lapse microscopy of ECL t*ssB1-gfp* Δ*rhsB rhsA-250* cells (CH12612).

Movie S9. Time-lapse microscopy of ECL t*ssB1-gfp* Δ*rhsB rhsA-966* cells (CH12613).

Movie S10. Time-lapse microscopy of ECL *tssB1-gfp* Δ*rhsA* Δ*rhsB* cells (CH12594).