Supplementary Materials for

Structural basis for effector recognition by an antibacterial Type IV Secretion System

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Materials and Methods

Bacterial strains and cloning. Oligonucleotides, plasmids and bacterial strains utilized in this study are described in Tables S6, S7 and S8. The cloning procedures were performed using PCR (Phusion polymerase, Thermo Scientific), cleavage by restriction enzymes, ligation reactions, and Gibson Assembly reactions (NEB) (Table S6). Mutagenesis reactions were performed with Quik Change II XL (Agilent) using pET28a-XAC2609HIS-311-431, pBRA-XAC2609 (1) and pBRA-XAC3633-XAC3634 plasmids as templates. Single colonies of X. citri strains transformed with pBRA-derived or pBBRGFP-derived vectors were selected on LB-agar plates containing 100 µg/mL spectinomycin or 20 µg/mL gentamicin, respectively. Liquid cultures of X. citri strains were grown as pre-inoculum in 2xTY (2 x tryptone and yeast extract) media containing the appropriate antibiotics at 28°C and 200 RPM for 12-16 hours and then diluted 100-fold in the specific media (see below). Antibiotics were maintained throughout all steps of growth and preparation of cell cultures. E.coli BL21(DE3) RP cells (Novagen) transformed with pET28- or pET11a-derived vectors (Novagen) were selected by growth on LB agar plates containing kanamycin (50 µg/mL) or ampicillin (150 µg/mL), respectively.

The X. citri Δ7Δ2609-GFP strain construction was based on the sequential deletions of the X-Tfi/X-Tfe pairs (except for the XAC2610 X-Tfi) genes from the X. citri genome(1–5). Deletions of each X-Tfi/X-Tfe pair were achieved using two-step allelic exchange with the pNPTS138a suicide vector (1) encoding approximately the 1 Kb regions both upstream to the X-Tfi and downstream to the X-Tfe. Table S6 describes the primers and pairing regions used for each deletion. This strain has a total of eight deletions which were introduced in the following order: 1) ΔXAC2885/XAC2884; 2) ΔXAC0574/XAC0573; 3) ΔXAC0097/XAC0096; 4) ΔXAC3634/XAC3633; 5) ΔXAC1918/XAC1917; 6) ΔXAC0467/XAC0466; 7) ΔXAC4264/XAC4263/XAC0462; 8) ΔXAC2609::msfGFP. For the 8th deletion, the xac2609 gene was replaced with the msfGFP gene (6), which allows the strain to be distinguished from target cells during time-lapse fluorescence microscopy, as has been described for a similar strain, Δ8Δ2609-
GFP, which has one additional X-Tfe/X-Tfi pair (ΔXAC3266/XAC3267) deleted (4) (Table S9). All plasmids were confirmed by DNA sequencing.

**Yeast two hybrid assays.** Yeast two-hybrid assays were carried as previously described (2, 7). The VirD4 all alpha domain (196-355), coded by the pOBD_VirD4_AAD vector, was used as a bait to screen a prey library of *X. citri pv. citri* 306 genomic DNA fragments cloned in the pOAD vector (2, 7). Screening was performed by selecting isolated colonies of transformed *Saccharomyces cerevisiae* strain PJ694 that grew in media lacking tryptophan, leucine, histidine and adenine followed by isolation and sequencing of the pOAD-derived plasmids, as previously described (2, 7).

**Protein expression, purification, SEC-MALS, and Analytical-SEC Analyses**

Protein expression conditions utilized in this study are described in Table S7. Colonies of *E. coli* BL21(DE3) cells transformed with specific plasmids were typically inoculated in 5 mL of 2xTY medium and grown overnight (37°C, 200 rpm), then diluted in 500 mL 2xTY. After reaching OD (600nm) = 0.7, the culture temperature was adjusted to the one used for induction (Table S7), IPTG was added to a final concentration of 0.5 mM and induction followed as described in Table S7. Samples isotopically labeled with $^{15}$N and $^{13}$C were induced using the same protocol except that 2xTY was substituted with M9 medium containing 20 mM $^{15}$NH$_4$Cl, 50 mM Na$_2$HPO$_4$, 20 mM KH$_2$PO$_4$, 10 mM NaCl, 2 mM MgSO$_4$, 20 mM $^{12}$C-glucose or $^{13}$C-glucose and 0.1 mM CaCl$_2$. Cells were collected by centrifugation and lysed with a French press at 5°C in lysis buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5% glycerol). The soluble fractions were recovered after centrifugation for 45 min at 25000 g. Chromatography purification was carried out at room temperature using an FPLC AKTA system (Cytiva), and each step was monitored by absorbance at 280 nm and 220 nm. Protein quantification was estimated by absorbance at 280 nm and the specific protein extinction coefficient or using the Bio-Rad DC Protein kit assay at 750 nm. N-terminal polyhistidinyl tagged proteins (Table S7) were first purified by affinity chromatography using HiTrap Ni$^{2+}$-chelating resin (Cytiva) equilibrated with 20 mM Tris-HCl buffer (pH 8.0), 200 mM NaCl, 20 mM imidazole, and 2% (v/v) glycerol. Elution was done using a 20 to 500 mM imidazole gradient. The XAC2609$_{XVIPCD}$ produced
from the expression of the pET11a vector was purified using Q-Sepharose anion-exchange chromatography (Cytiva) as previously described(1, 2). This XAC2609XVIPCD construct that does not present an N-terminal polyhistidine tag (Table S7) was used to perform Pulldown assays (see below), SAXS and Analytical-SEC experiments. Copurification assays of VirD4His-AAD and XAC2609XVIPCD were performed by mixing the lysates of cells expressing each protein individually and applying the same protocol for the purification of polyhistidine tagged proteins described above. Purified His-tagged proteins (Table S7) were subjected to cleavage of their N-terminal tags overnight at 4 °C according to the "Thrombin CleanCleave" kit (Sigma). Samples were then submitted to a final purification step by passage through a Superdex 75 26/600 size exclusion column (Cytiva) in Tris buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 2% glycerol). The VirD4AAD-XAC2609XVIPCD complex was purified by His-tag affinity chromatography followed by the removal of the VirD4His-AAD N-terminal His-tag using thrombin and a size exclusion chromatography as the final step. The VirD4AAD-XAC2609XVIPCDΔ20 complex was obtained by mixing purified samples followed by size exclusion column chromatography (Superdex 75 26/600). The molecular masses of VirD4AAD, XAC2609XVIPCD, XAC2609XVIPCDΔ20, and the complexes formed by VirD4AADXAC2609XVIPCD and VirD4AAD-XAC2609XVIPCDΔ20 were estimated using SEC-MALS (size-exclusion chromatography coupled to multi-angle light scattering). SEC-MALS was performed using a Superdex 75 10/300 GL (Cytiva) coupled to a Wyatt MALS detector and data were processed using the ASTRA software with a Zimm light scattering model and refractive index increment dn/dc = 0.185 mL/g. SEC-MALS and analytical SEC were performed using buffers at pH 8.0 (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 2% (v/v) glycerol), pH7.0 (20 mM Tris-HC (pH 7.0), 200 mM NaCl, 2% (v/v) glycerol), pH 6.0 (20 mM Mes-OH (pH 6.0), 200 mM NaCl, 2% (v/v) glycerol) and pH 4.5 (20 mM Na-acetate (pH 4.5), 200 mM NaCl, 2% (v/v) glycerol) with protein samples ranging from 1 to 2 mg/ml and a flow rate of 0.5 mL/min. Supernatants of cell lysates of BL21-CodonPlus (DE3) RIL (Agilent) RIL in buffer (50 mM Tris pH 8.0 200 mM NaCl 20 mM Imidazole and 10 % glycerol) expressing HIS-XAC2609XVIPCD, HIS-XAC2609XVIPCDF375A-V377A, HIS-XAC2609XVIPCDΔ20, PcfG-His, His6-PcfF, His-VirD2, as baits, and VirD4AAD as prey were mixed and washed (20 cv) and eluted (10 cv) through a HiTrap Chelating HP (Cytiva) (5 mL) immobilized with cobalt column
using 20 mM to 50 mM Imidazole gradient. Same approach was performed using the supernatants of cell lysates coexpressing the VirD4(83-557) as a prey and HIS-XAC2609XVIPCD derivatives a bait. Interactions between the proteins were also confirmed by Tricine-SDS-PAGE. Quantitative analysis was performed with the Image Studio Lite (version 4.0) software package.

**Isothermal titration calorimetry.** Isothermal titration calorimetry experiments were performed in a MicroCal VP-ITC calorimeter (Malvern). Typically, VirD4AAD (10 to 20 µM) was injected into the titration cell and His-tagged XAC2609XVIPCD samples into the syringe. Aliquots of 5 to 10 µL of titrants at 100 to 250 µM were gradually added from the syringe to the cell. ITC assays were performed at 298 K. Buffers were exchanged to 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 2% (v/v) glycerol using PD-10 Desalting Columns (Cytiva) or Amicon Ultra (Millipore) Centrifugal devices. Heat curves integrals were obtained using the Origin Microcal software and dissociation constants (Kd) were calculated over 200 fitting iterations with the ITC Data Analysis software. The Kd values represent the mean of two to four independent assays and errors are reported as standard deviations.

**Small Angle X-ray Scattering (SAXS).** SAXS experiments were carried out at the SAXS 2 beamline of the Brazilian Synchrotron Light Laboratory (LNLS). X-ray scattering was collected using 1.55 Å radiation and a MAR CCD 165 detector (MAR Research) at a distance of 935.5 mm from the sample. The scattering vector q (q = 4µ/λsin(ө), where 2ө is the scattering angle) interval was set between 0.016 Å⁻¹ and 0.356 Å⁻¹. SAXS measurements were collected for 30 to 600 seconds at 2 to 3 protein concentrations on dialyzed samples of VirD4AAD (1, 2 and 5 mg/ml), XAC2609XVIPCD (2, 4 and 8 mg/mL) and the VirD4AAD-XAC2609XVIPCD co-purified complex (1 and 2 mg/mL). SAXS data were also obtained for the dialysis buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5% (v/v) glycerol). The SAXS curves were normalized by protein concentration to monitor radiation damage or aggregation effects. No radiation or aggregation effects were evidenced. Data reduction, analysis and normalization by concentration was performed with the ATSAS package (8). SAXS curves represent the mean of two to three independent assays.
normalized by the concentration and errors are reported as standard deviations. Graphs were generated using Origin data analysis software.

**NMR Spectroscopy and Structure Calculation.** NMR experiments were performed on a Bruker Avance III 800 MHz NMR spectrometer at 25°C equipped with a TCI cryogenic probe. All NMR samples consisted of 15N labeled or 15N and 13C doubly labeled XAC2609_{XVIPCDΔ20} at a concentration of 1.6 mM in NMR buffer (20 mM Tris-HCl pH 8, 100 mM NaCl, 0.1% glycerol, 7% D$_2$O, 0.05% sodium azide) unless otherwise stated. Backbone resonance assignments were obtained for most residues, except for 318-319, 333, 340, 369-370, 381 - 382, 397 - 399, and 403 - 408. These assignments were obtained from the analysis of a set of BEST-TROSY triple resonance NMR experiments, HNCA/CBCA(CO)NH, HNCA/HN(CO)CA, HNCO/HN(CA)CO, implemented using NMRLib(9). Side-chain resonances were obtained from the analysis of 3D-(H)CCH (TOCSY) and 3D-H-(C)CH TOCSY experiments. All triple resonance and TOCSY NMR experiments were recorded using non-uniform sampling, and reconstructed using MddNMR(10) or the IST approach with the poisson-gap method to generate the sampling schedule (11). All NOESY experiments were recorded using a mixing time of 100 ms. The following NOE experiments were recorded in H2O: 3D 15N NOESY-HSQC, 13C NOESY-HSQC and 1H-1H 2D NOESY. NOEs from aromatic residues were obtained from the analysis of a 13C NOESY-HSQC experiment recorded as a 2D plane and with the 13C carrier frequency centered in the aromatic region. Additionally, H(C)CH-TOCSY, (H)CCH-TOCSY, 13C NOESY-HSQC, 15N NOESY-HSQC, and 2D NOE spectra were collected using a lyophilized sample resuspended in deuterated buffer. The NMR titration was carried out by recording 1H-15N-HSQC experiments of $^{15}$N-$^{13}$C-XAC2609$_{XVIPCDΔ20}$ at a concentration of 160 µM in the absence or presence of VirD4$_{AAD}$ at 40 µM, 80 µM, 120 µM, 160 µM, 320 µM protein concentrations. Ratios of the 1H-15N-HSQC peak heights at the last titration point (XAC2609$_{XVIPCDΔ20}$ [160 µM]: VirD4$_{AAD}$ [320 µM]) with respect to the first titration point (XAC2609$_{XVIPCDΔ20}$ [160 µM] ) were mapped on the NMR structure of XAC2609$_{XVIPCDΔ20}$. NMR spectra were processed with NMRPipe (12) and analyzed with CcpNmr Analysis (13), data analysis and graphs were made using Origin(2020).
Automated NOE assignment and structure calculation were performed with the Ariaweb server at [https://ariaweb.pasteur.fr/](https://ariaweb.pasteur.fr/)(14), by simulated annealing molecular dynamics in torsion angle space(15), based on the assignments for 81.9% of backbone resonances, and 73.8% of 1H side chain resonances, 5462 manually picked NOESY crosspeaks and 154 dihedral angle restraints obtained using Talos-N (16). A total of 15 iterations were performed, for each interaction 50 structures were calculated. Network anchoring was not used, and all default options were maintained except for the use of spin diffusion correction in iterations 3-15, log-harmonic potentials (17), and automatic restraints weighting. A total of 2383 NOEs were assigned and used in the calculation. The 20 lowest energy models were refined in explicit solvent and chosen for the ensemble of the XAC2609\(_{\text{XVIPCD}}\)\(\Delta_{20}\). The precision of the final ensemble is 0.69 Å for the backbone coordinates within residues 316 - 393. An average of 32.9 distance restraints were violated by more than 0.5 Å, however, they are not consistently violated in more than 50% of the conformers. When all distance restraints violations are considered, the average violation is 0.148 Å ± 0.026 Å (Table S3). The stereochemical quality of the final ensemble was examined with the Ramachandran plot, which showed 97.2% of residues in the most favoured and allowed regions, 1.3% in the generously allowed region, and 1.4% in the disallowed region.

**Thermal denaturation monitored by circular dichroism (CD).** CD assays were performed on a Jasco J-815 Spectropolarimeter with a 0.5 mm optical path length quartz cuvette. Samples of XAC2609, XAC2609(1-306), and the XAC2609\(_{\text{XVIPCD}}\) at a concentration ranging from 0.5-0.75 mg/mL were diluted in buffer pH 8 (5 mM Na-Hepes, 20 mM Na\(_2\)SO\(_4\)) and pH 5 (5 mM Na-Acetate, 20 mM Na\(_2\)SO\(_4\)). CD spectra were typically acquired with 1 nm data pitch, 1 second of D.I.T, scanning speed at 50 nm/min ranging from 195 to 260 nm, with signal is the average of eight-time acquisitions for each assay. Thermal denaturation curves were monitored at 222 nm from 5°C to 70°C at 1°C/minute with a 30 second interval before each acquisition. The buffer spectrum was subtracted from the (protein + buffer) and smoothed by the Savitzky-Golay method. The normalized denaturation curve (% unfolded vs T) was estimated as described (18).
**CPRG bacteria-killing experiments.** To prepare *X. citri* cells for the assays, they were cultivated in 24-well plates for 12 to 14 hours in 2xTY medium at 28°C, 200 RPM, followed by three rounds of sedimentation (3 minutes, 5000 g) and resuspension with 2xTY before the assays. *E.coli* K12 strain MG1655 cells were prepared by growing overnight in 2xTY at 37 °C and 200 RPM, and diluted 100x in 2xTY supplemented with 100 µM IPTG to induce production of beta-galactosidase, cultivated for 3 hours at 37 °C, 200 RPM, and washed three times with 2xTY medium. CPRG bacteria-killing assays were performed as previously described(19), with some adaptations. Briefly, these experiments were carried out in a 96-well plate reader (SpectraMax Paradigm, Molecular Devices) at room temperature using U-shaped bottom 96-well plates (Corning Costar, catalog 3799). Each well was filled with 100 µL of agarose-CPRG medium (1.5 % (w/v) agarose, 40 µg/mL CPRG (Sigma-Aldrich), 2 mM CaCl$_2$, 100 µM IPTG) and allowed to dry for 2 hours at room temperature. Next, 5 µL of the mixtures of *X. citri* strains (OD$_{600nm}$ = 0.5) and *E. coli* MG1655 cells (OD$_{600nm}$ = 5) were spotted over the wells filled with agarose-CPRG medium. The plates were covered with a transparent seal and immediately loaded in the plate reader. Absorbance was monitored at 572 nm (A$_{572nm}$) at 10 minutes intervals for 8 hours setting the initial absorbance to zero. The signal background during the course of the experiment was estimated by the A$_{572nm}$ curve from a negative control containing only *E.coli* MG1655 cells. Data reduction was performed by subtracting the signal background from the experimental curves obtained for different *X. citri* strains. Normalization was then performed by dividing absorbance values at each time point by the A$_{572nm}$ at 480 minutes. Data reduction, first derivative determination and statistical analysis was performed using Origin.

**Colony growth competition assays.** Colony competition assays were performed as previously described(1, 5), with some modifications. *X. citri* strains used were wild type, ΔViB7 and Δ7Δ2609-GFP transformed with the empty pBRA plasmid and *X. citri* Δ7Δ2609-GFP transformed with pBRA-XAC2609 or pBRA-XAC3633-XAC3634. The competing strain was *E. coli* BL21(DE3)RIL ArcticExpress transformed with the vector pBBR(1)RFP, that confers cells a red color phenotype and kanamycin resistance. After washing with 2xTY, *X. citri* cultures were resuspended at OD$_{600nm}$ = 2 and *E. coli* at OD$_{600nm}$ = 0.2. Equal volumes of cell cultures were mixed, resulting in a final cell ratio of
**X. citri**: *E. coli* of 10:1. Five µL of this mixture were pipetted onto Luria-Bertani (LB) agar plates supplemented with spectinomycin (100 mg/mL) and incubated at 30°C for 36 hours. Finally, colonies were resuspended in 2 mL 2xTY and CFU/mL was monitored by serial dilution on LB agar plates supplemented with ampicillin or kanamycin for selection of *X. citri* or *E. coli*, respectively.

**Time lapse microscopy assays.** Microscopy assays were performed as previously described (1, 4), with modifications. Briefly, a Nikon Ti microscope equipped with a GFP filter (GFP-3035B-000-ZERO, Semrock), a RFP filter (Texas Red BrightLine, TXRED4040-B, Semrock) and a Plan APO lambda 100x 1.45 oil ofn25 ph3 DM objective were utilized for microscopy assays. Time-lapse microscopy images were taken every 10 minutes for 4 hours at 22°C. Microscopy chambers were built of a glass microscope slide (7.5 cm x 2.5 cm) attached to a double-sided tape (3M) (5.5 cm x 2.5 cm x 2 mm), forming a central rectangular aperture (3 cm x 5 mm x 2 mm) filled with LB-agar in which 1 µL of the mixed bacterial cultures were deposited, allowed to dry, and then closed with coverslips. Images were processed using the Nis Elements software (version 3.07; Nikon), analyzed with Fiji(20) and drifts corrected using the StakReg plugin(21).

**Micrococcus luteus** peptidoglycan hydrolysis assays. Peptidoglycan hydrolysis experiments were performed as previously described (1), with some modifications. Briefly, *M. luteus* cell wall suspensions (Sigma) at OD$_{650nm} = 0.7$ (1.2 mg/mL) in 50 mM sodium acetate (pH 5.0) and 2 mM CaCl$_2$ were incubated in triplicate for 2 hours at 30 °C with only buffer (negative control), 2 µM XAC3633(HIS-40-300), 1 µM XAC3634 (HIS-1-513), 1 µM XAC3634E35Q (HIS-1-513), or a 2 µM XAC3633(HIS-40-300) + 1 µM XAC3634(HIS-1-513) mixture. Reactions were quenched by adding 500 mM sodium carbonate. Peptidoglycan hydrolysis was monitored as a decrease in OD$_{650nm}$.

**Bioinformatics.** Search for X-Tfes and taxonomic analysis were performed using the sequence of residues 311-431 of XAC2609 as a query in PSI-Blast(22) against the non-redundant protein sequence database (all non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects). Two iterations were performed, with the e-value threshold set to $1 \times 10^{-6}$, and the
hit list size limited to 10000 sequences. Then, the last 150 residues were extracted from each hit, redundant sequences were removed using Jalview (2.11)(23) and aligned with Mafft(24). Truncated sequences and potential outliers were excluded from the XVIPCD final data set. A total of 4756 sequences were selected for WebLogo analysis(25) and XVIPCD N-terminal and C-terminal alignments were obtained using the XAC2609XVIPCD sequence as reference in Jalview, followed by compressing vectorial images with Illustrator. Conservation mapped to the structure of the XAC2609XVIPCDΔ20 was achieved using the Mutalign Viewer tool in Chimera(26). All images of protein structures were prepared using Chimera(26).

**Western-blot.** Protein samples coeluted from the pull-down assay of His-VirD4AAD-XAC2609XVIPCD were separated by SDS-PAGE 16% and transferred to a nitrocellulose membrane using a semi-dry blot system at 60 mA for 1 hour. After transfer, the membrane was blocked (TBS buffer 10% non-fat milk, 50 rpm, 16 hours) and then incubated with anti-XAC2609 rabbit serum(1) (1:1000 dilution, two hours, 50 rpm), washed (five steps: PBS buffer, 10% non-fat milk, 0.1% tween 20, 5 minutes, 50rpm), incubated with IRDye 800CW goat anti-rabbit IgG (LI-COR Biosciences) at 1:15,000 dilution. Anti-polyhistidine-alkaline phosphatase conjugated (Sigma A5588) (1:20000 dilution, two hours, 50 rpm), was incubated for one hour, washed and revealed with 22.5 ul de BCIP (50 mg/ml) e 30 ul de NBT (75 mg/m 70 % dimethylformamide) in buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl). Images were acquired with ChemiDoc System MP (BioRad).
Supplementary Text

XVIPCD sequences are not similar to any other protein families with known structures (see Supplementary Text). We therefore used the ensemble of twenty XAC2609\_XVIPCD\_Δ20 solution structures to search for proteins with structural similarities using the Dali server(34). The most significant hit, the small RNA-binding protein HutP (PDB IDs 1VEA and 1WPU) that regulates the histidine utilization operon in Bacillus subtilis(35), has an \(αβαβββ\) topology, whose last five secondary structures fold with the same \(αβββ\) topology as that of XAC2609\_XVIPCD\_Δ20. However, the structural alignment with XAC2609\_XVIPCD\_Δ20 has a relatively low Z-score (4.0) and very low sequence identity (8%).

Supplementary Figures
Figure S1. Sequence alignment of Type IV Coupling proteins. Above: Schematic of domain architectures of VirD4 from A. tumefaciens (VirD4 A.tu), VirD4 from X. citri (VirD4 X. ci), TrwB from the E. coli plasmid R388 (TrwB R388), and PcfC from the Enterococcus faecalis plasmid pCF10 (PcfC pCF10). Transmembrane domain (beige), nucleotide binding domain (brown), all-alpha domain (blue), C-terminal extension (green). Below: Protein sequence alignment using T-coffee(27). The all-alpha domain (AAD) observed in the crystal structure of TrwB(28) and predicted for A. tumefaciens VirD4 (29), PcfC (29) and X. citri VirD4 are in blue bold type. Sequences within the AADs predicted to form α-helices are highlighted in rose.
**Figure S2. Purification of the VirD4\text{AAD}-XAC2609\text{xVIPCD complex.}** (A) Tricine 16% SDS-PAGE of fractions obtained for the expression and purification of the VirD4\text{HIS-AAD}-XAC2609\text{XVIPCD} complex. Total cell lysates of *E. coli* cells before (NI) and after induction of expression of XAC2609\text{XVIPCD} (I1) or VirD4\text{HIS-AAD} (I2). Soluble fraction of combined total cell lysates after VirD4\text{HIS-AAD} and XAC2609\text{XVIPCD} induction (S). Purification of the VirD4\text{HIS-AAD}-XAC2609\text{XVIPCD} complex by Ni\textsuperscript{2+}-affinity chromatography: fraction of unbound proteins eluted during the column washing step (FT) and during the 20-500 mM imidazole gradient (1, 2, 3). (B) Detection of XAC2609\text{XVIPCD} and VirD4\text{HIS-AAD} in the purified fractions by western blot. Immunodetection was performed using primary anti-XAC2609 antibody (α-XAC2609) and anti-polyhistidine (α-His-VirD4\text{AAD}) antibody. (C) Analytical size exclusion chromatography of XAC2609\text{XVIPCD}, XAC2609\text{XVIPCDΔ20}, VirD4\text{AAD}, VirD4\text{AAD}-XAC2609\text{XVIPCD} and VirD4\text{AAD}-XAC2609\text{XVIPCDΔ20} at pH 8.0 (black; 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 2% glycerol), pH 7.0 (yellow; 20 mM Tris-HCl pH 7.0, 200 mM NaCl, 2% glycerol), pH 6.0 (green; 20 mM Mes-OH pH 6.0, 200 mM NaCl, 2% glycerol), and pH 4.5 (blue; 20 mM sodium acetate pH 4.5, 200 mM NaCl, 2% glycerol). (D) SEC-MALS analysis of the purified recombinant XAC2609\text{XVIPCD}, XAC2609\text{XVIPCDΔ20}, VirD4\text{AAD}, and the VirD4\text{AAD}-XAC2609\text{XVIPCD} complex at pH 4.5 (20 mM sodium acetate (pH 4.5), 200 mM NaCl, 2% glycerol). Normalized absorbance at 280 nm (black line, left axis) and molecular mass distribution (gray dots, right axis) are shown as a function of elution volume (mL). Representative elution profiles are shown for each sample.
VirD4(83-557) + HIS-XAC2609 derivatives

**A**

| MW | S | FT | Elu |
|----|----|----|-----|
| (kDa) | | | |
| 250.0 | | | |
| 150.0 | | | |
| 100.0 | | | |
| 75.0 | | | |
| 50.0 | | | |
| 37.0 | | | |
| 20.0 | | | |
| 25.0 | | | |
| 15.0 | | | |
| 10.0 | | | |

**B**

**C**

relative signal intensity

0% 50% 100%

S FT ELU S FT ELU S FT ELU S FT ELU S FT ELU
Figure S3. XAC2609XVIPCD interaction with the cytoplasmic portion of VirD4. (A) Coomassie-stained Tricine-SDS-PAGE of the cobalt affinity pulldown assay between distinct HIS-tagged XAC2609XVIPCD constructs (*) and the cytoplasmic portion of VirD4 (VirD4(83-557) (#)). E. coli BL21De3 cells co-expressing VirD4(83-557) + empty pET28a vector (VirD4(83-557) only), VirD4(83-557) + HIS-XAC2609xVIPCD (xVIPCD), VirD4(83-557) + HIS-XAC2609xVIPCDΔ20 (xVIPCDΔ20), VirD4(83-557) + HIS-XAC2609xVIPCDF375A/V377A (xVIPCDF375A V377A), VirD4(83-557) + HIS-XAC2609(392-431) (392-431). Lane labels: S: soluble fractions of the supernatants before application to Co²⁺ affinity column; FT: flow-through fraction with unbound proteins obtained by washing with buffer A (50 mM Tris-HCl pH8, 200 mM NaCl, 20 mM Imidazole, 10% glycerol); ELU: bound protein fraction obtained by elution with an imidazole gradient (20 mM - 500mM); MW: molecular weight markers. (B) Scanned image of the coomassie-stained gel shown in (A) employed for quantification of the 50 KDa band corresponding to VirD4(83-557) (highlighted with the blue rectangle). (C) Relative signal intensities for the highlighted area measured in (B).
Figure S4. NMR analysis of XAC2609XVIPCDΔ20. (A) $^1$H-$^{15}$N-HSQCs spectra of $^{15}$N-labeled XAC2609XVIPCDΔ20 (250 µM, 25 °C) at pH 4.5 (20 mM sodium acetate, 20 mM NaCl, 0.1% glycerol), pH 6.0 (20 mM Mes-NaOH, 20 mM NaCl, 0.1% glycerol), pH 7.0 (20 mM Tris-HCl, 20 mM NaCl, 0.1% glycerol), and pH 8.0 (20 mM Tris-HCl, 20 mM NaCl, 0.1% glycerol). (B) Superposition of $^1$H-$^{15}$N-HSQC spectra of $^{15}$N-XAC2609XVIPCD (red) and $^{15}$N-XAC2609XVIPCDΔ20 (black). (C) *Left vertical scale:* NMR chemical shift-based TALOS-N secondary structure prediction (30): alpha helix, red bars; beta sheet, blue bars. *Right vertical scale:* amide heteronuclear $[^1]$H-$^{15}$N NOE (HetNOE) of $^{15}$N-labelled-XAC2609XVIPCDΔ20 (black dots) and the RCI chemical shift order parameter predicted by TALOS-N (gray dots) as a function of the protein sequence. (D) XAC2609XVIPCDΔ20 structure showing Pro400 and residues with which it makes close contacts (as detected by NOEs). Pro400, Asp372, Phe390, Asp392, Arg393 and Asn394 are shown as cyan colored stick models (hydrogens are not shown). For the rest of the molecule, α-helices are red, β-sheets are yellow and loops are green. For clarity, residues 396-411 are not shown.
**Figure S5. Circular dichroism (CD) analysis of the X-Tfe\textsuperscript{XAC2609}.** (A) Schematic model of X-Tfe\textsuperscript{XAC2609} showing the glycohydrolase 19 domain (GH19), peptidoglycan-binding (PG binding) domain and the XVIPCD. CD spectra of the X-Tfe\textsuperscript{XAC2609} residues 1-431, XAC2609(1-431); residues 1-206 XAC2609(1-306); and residues 311-431, XAC2609\textsubscript{XVIPCD}. CD spectra were obtained at 25°C before denaturation assay (solid black line), at 70°C (red line) and at 25°C after denaturation assay (dotted black line) at pH 5.0 (5 mM Na-Acetate, 20 mM Na\textsubscript{2}SO\textsubscript{4}) and at pH 8.0 (5 mM Hapes-Na, 20 mM Na\textsubscript{2}SO\textsubscript{4}).

(B) Thermal denaturation assay monitored by CD. Mean residual ellipticity ([Θ]) at 222 nm of XAC2609(1-431) (square), XAC2609(1-306) (circle) and XAC2609\textsubscript{XVIPCD} (triangle) at pH 5.0 (gray) and pH 8.0 (black). Thermal denaturation and assays were performed from 5°C to 70°C, with data collected at 1°C intervals. Ramp speed was 1°C/min with 30 seconds equilibration time before reads. (C) Normalized denaturation curves obtained using the data shown in (B) except for XAC2609\textsubscript{XVIPCD} at pH 5.0 which did not present a well defined unfolding transition. The fraction of unfolded protein at each temperature was calculated as described (18).
Figure S6. $^{15}\text{N}^{-1}\text{H}$ HSQC spectrum and assignment of $^{15}\text{N}^{-13}\text{C}$-labelled $\text{XAC2609}_{\text{VIPCD}}\Delta 20$ at pH 8.0. The crowded region in the center of the spectrum (small box) is amplified in the large box at the top left.
Figure S7. Ribbon representation of the models of XVIPCD domains from X-Tfes selected from a wide range of bacterial species. The figure shows the first top-ranked models for each prediction obtained using the RaptorX (31, 32) server. Labels provide the access code for the protein sequence (GenBank or Kegg) with the residues of the domain given in lowercase parentheses and the bacterial species in square brackets. Only the αβββ regions of each model are shown.
Figure S8. VirD4AAD-induced perturbations in the $^{15}$N-$^1$H HSQC spectrum of XAC2609XVIPCDΔ20. (A) $^{15}$N-$^1$H HSQC spectrum of $^{15}$N-labelled XAC2609XVIPCDΔ20 (160 µM) was measured on its own (160:0) and in the presence of increasing concentrations of unlabelled VirD4AAD: 0 µM (black), 40 µM (yellow), 80 µM (green), 120 µM (cyan), 160 µM (purple), 320 µM (red). Molar ratios of the two proteins are shown. (B) Superposition of the $^{15}$N-$^1$H HSQC spectra shown in A. (C) Amplification of the central region of the superposed spectra within the square in (B). All spectra were collected at 25°C in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% glycerol.
Figure S9. Titration of VirD4\textsubscript{AAD} by XAC2609, XAC2609\textsubscript{XVIPCD} and its mutants, monitored by isothermal calorimetry. (A). Representative titrations for each mutant are shown. Data points represent the heat released after each injection of XAC2609, XAC2609\textsubscript{XVIPCD} or its mutants. Assays were performed at 298 K in 20 mM Tris-HCl pH8, 200 mM NaCl, 2% glycerol. All XAC2609\textsubscript{XVIPCD} fragments used in these experiments have an N-terminal His-tag. Dissociation constants (K\textsubscript{D}) were calculated as described in Materials and Methods and are listed in Supplementary Table 4. (B) Ribbon representation of the XAC2609\textsubscript{XVIPCD}\textsubscript{Δ20} structure with mutated residues within the ααβββ region shown as space-filling models (green). (C) Isothermal titration calorimetry of titration of X. citri VirD4\textsubscript{AAD} with HIS-PcfF and His-PcfG from E. faecalis and His-VirD2 from A. tumefaciens (29).
Figure S10. Pull-down assays to detect *Xanthomonas citri* VirD4<sub>AAD</sub> interactions. Coomassie blue stained tricine-SDS-PAGE of the Co<sup>2+</sup>-affinity pulldown assays to detect interactions between the *X. citri* VirD4<sub>AAD</sub> and distinct HIS-tagged T4SS substrates: His-XAC2609<sub>XYIPCD</sub>, His-XAC2609<sub>XYIPCD</sub>F375A/V377A, *E. faecalis* HIS-PcfF, *E. faecalis* PcfG-His and *A. tumefaciens* His-VirD2. VirD4 and His-tagged substrates were expressed separately, equal numbers of cells combined and lysed together. (+) and (-) indicates the presence and absence, respectively, of *X. citri* VirD4<sub>AAD</sub> in each assay. Lane labels: S, soluble bacterial cell lysates before application to the Co<sup>2+</sup> affinity column; FT, unbound protein fraction obtained by washing with buffer A (50 mM Tris-HCl pH8, 200 mM NaCl, 20 mM Imidazole, 10% glycerol); ELU, bound proteins obtained by eluting with a 20 mM - 500 mM imidazole gradient; MW, molecular weight markers.
Figure S11. CPRG bacterial competition assay. Mixed cultures of *X. citri* strains and *E. coli K-12 MG1655* cells (constitutively expressing β-galactosidase) were grown at 25°C in 96 well plates containing agar media supplemented with CPRG. Absorbance at 572 nm was measured every 10 min for 480 minutes. *X. citri* strains used in each assay are indicated in each panel. Error bars represent the standard error from three to five independent measurements.
Figure S12. Correlation of the effect of XVIPCD mutations on XVIPCD-VirD4_{AAD} binding affinity and efficiency of X-Tfe transfer into the target cell. Binding affinity of XAC2609_{XVIPCD} mutants for VirD4_{AAD} is expressed on a discontinuous scale of K_D values (left vertical axis) as determined by ITC assays (Table S4). Relative killing efficiencies of *X. citri* Δ7ΔXAC2609-GFP strains carrying plasmids expressing X-Tfe^{XAC2609} (central vertical axis) or X-Tfe^{XAC3634} (right vertical axis) with mutations in their XVIPCDs. Relative killing efficiencies are derived from Figures 4D and 5G, respectively.
Table S1. Molecular Mass estimated by SEC-MALS and Analytical SEC.

| Protein                  | Theoretical Mass | SEC (mL) | MALS (KDa) | SEC (mL) | MALS (KDa) | SEC (mL) | MALS (KDa) | SEC (mL) | MALS (KDa) |
|--------------------------|------------------|----------|------------|----------|------------|----------|------------|----------|------------|
| XAC2609 XVIPCD           | 13.7 KDa         | 11.4     | 11.0 +/- 2%| 12.0     | 11.0 +/- 2%| 12.2     | 20.0 +/- 3%| 11.8;    | 12.8 ND    |
| VirD4AAD                 | 18.0 KDa         | 11.5     | 11.0 +/- 2%| 12.0     | 11.0 +/- 2%| 12.4     | ND         | 11.5;    | 13.2 ND    |
| VirD4AAD-XAC2609 XVIPCD  | 31.7 KDa         | 12.6     | 11.6 +/- 5%| 12.3     | 11.6 +/- 5%| 12.7     | 20.7 +/- 3%| 11.0     | 34.0 +/- 1%| 11.2     | 30.1 +/- 4%|
| VirD4AAD-XAC2609 XVIPCDΔ20| 29.5 KDa         | ND       | ND         | ND       | ND         | 10.9     | ND         | 11.2     | ND         |

Theoretical molecular mass after purification and cleavage of the N-terminal histidine tail is shown. Estimated molecular mass assuming the formation of a heterodimer in solution (18.0 KDa + 13.7 KDa). Estimated molecular mass assuming the formation of a heterodimer in solution (18.0 KDa + 11.5 KDa). SEC elution volume of the peaks shown in Supplementary Figure 2C. MALS: Molecular mass estimated in accordance with mass distribution shown in Figure 1 A and Supplementary Figure 2D. *: †SEC elution volume of the first* and the second † peaks shown in Supplementary Figure 2C indicating the dissociation of the VirD4AAD-XAC2609 XVIPCD and VirD4AAD-XAC2609 XVIPCDΔ20 complexes at acidic pHs (pH 4.5 and pH 6.0). Value not determined (ND).
Table S2. Two-hybrid assays to identify protein-protein interactions between VirD4\textsubscript{AAD} (residues 197-355) and preys derived from a library of protein fragments coded by the \textit{Xanthomonas citri str 306} genome.

| Bait  | Specific preys (\textit{X. citri gene}) | number of independent prey clones | initial codon of smallest prey |
|-------|----------------------------------------|-----------------------------------|--------------------------------|
| VirD4\textsubscript{AAD} | XAC0151 | 2 | 6 |
|       | XAC0096 | 5 | 354 |
|       | XAC3266 | 1 | 656 |
|       | XAC2885 | 1 | 78 |

* Reference: (2)
Table S3. NMR data collection and structural refinement statistics for XAC2609\textsubscript{XVIPCDΔ20}

| NMR distance and dihedral constraints | \textbf{XAC2609\textsubscript{XVIPCDΔ20}} |
|---------------------------------------|---------------------------------|
| Distance constraints                  | \multicolumn{1}{c}{2383}         |
| Total NOE                             |                                 |
| Intra-residue                         | 750                             |
| Inter-residue                         |                                 |
| Sequential \((i - j) = 1\)            | 390                             |
| Medium-range \((i - j) < 4\)          | 322                             |
| Long-range \((i - j) > 5\)            | 578                             |
| Ambiguos                              | 343                             |
| Total dihedral angle restraints       |                                 |
| \(\phi\)                             | 77                              |
| \(\psi\)                             | 77                              |

**Structural refinement statistics**

| Violations (mean and s.d.)             | \multicolumn{1}{c}{0.148 ± 0.026} |
| Distance constraints (Å)               |                                 |
| Dihedral angle constraints (°)         | 0.000 ± 0.000                    |
| Max. distance constraint violation (Å) | 0.1                             |
| Deviations from idealized geometry     | \multicolumn{1}{c}{0.003 ± 0.0001} |
| Bond lengths (Å)                       |                                 |
| Bond angles (°)                        | 0.45 ± 0.014                     |
| Impropers (°)                          | 1.29 ± 0.072                     |
| Average pairwise r.m.s. deviation* (Å) | \multicolumn{1}{c}{1.67 ± 0.42}  |
| Heavy                                 |                                 |
| Backbone                              | 0.69 ± 0.20                      |

*Pairwise r.m.s. deviation was calculated among 20 refined structures for XVIPCD\textsubscript{XAC2609Δ20} residues 315-393.
Table S4. Bacterial strains that code for a putative X-T4SSs and X-Tfes

| Organism                                | Organism                  | Organism                  |
|-----------------------------------------|---------------------------|---------------------------|
| [Pseudomonas] geniculata ATCC 19374 = JCM 13324 | Luteibacter pinisoli      | Lysobacter sp. cf310      |
| [Pseudomonas] geniculata N1             | Luteibacter rhizovicinus  | Lysobacter sp. CHu50b-3-2 |
| Acinetobacterbaumannii                  | Luteibacter rhizovicinus DSM 16549 | Lysobacter sp. HDW10      |
| Alcaligenaceae bacterium                | Luteibacter sp. 22Crub2.1 | Lysobacter sp. lI4       |
| Alphaproteobacteria bacterium           | Luteibacter sp. 329MFSha  | Lysobacter sp. N42       |
| Bacillus sp. SRB_336                    | Luteibacter sp. OK325     | Lysobacter sp. Root494    |
| bacterium AM6                           | Luteibacter sp. Sphag1AF  | Lysobacter sp. Root559    |
| bacterium M00.F.Ca.ET.141.01.1.1        | Luteibacter sp. UNC138MFCol5.1 | Lysobacter sp. Root604   |
| bacterium M00.F.Ca.ET.163.01.1.1        | Luteibacter sp. UNC MF331Sha3.1 | Lysobacter sp. Root667   |
| bacterium M00.F.Ca.ET.177.01.1.1        | Luteibacter sp. UNC MF366Tsu5.1 | Lysobacter sp. Root76   |
| bacterium M00.F.Ca.ET.199.01.1.1        | Luteibacter yeojuensis    | Lysobacter sp. Root916    |
| Betaproteobacteria bacterium HGW-16     | Luteimonas aestuarii      | Lysobacter sp. Root96     |
| Betaproteobacteria bacterium HGW-9      | Luteimonas arsenica       | Lysobacter sp. Root983    |
| Burkholderiales bacterium GWF1_66_17   | Luteimonas gilva          | Lysobacter sp. SJ-36      |
| Burkholderiales bacterium rifcsphighO2_12_FULL_67_38 | Luteimonas granuli       | Lysobacter sp. TY2-98     |
| Dyella ginsengisoli                    | Luteimonas huabeiensis    | Lysobacter sp. UKS-15     |
| Dyella jiangningensis | Luteimonas mephitis | Lysobacter sp. URHA0019 |
| Dyella marenis | Luteimonas padinae | Lysobacter sp. yr284 |
| Dyella soli | Luteimonas sp. 83-4 | Lysobacter sp. zong2i5 |
| Dyella sp. 333MFSha | Luteimonas sp. H23 | Lysobacter xinjiangensis |
| Dyella sp. 4MSK11 | Luteimonas sp. MC1782 | Morococcus cerebrosus |
| Dyella sp. AD56 | Luteimonas sp. Sa2BVA3 | Neisseria dentiae |
| Dyella sp. AtDHG13 | Luteimonas sp. YD-1 | Neisseria flavescens SK114 |
| Dyella sp. DHOA06 | Luteimonas terricola | Neisseria lactamica |
| Dyella sp. OAE510 | Lutibacter sp. SG786 | Neisseria meningitidis |
| Dyella sp. OK004 | Lysobacter alkalisoli | Neisseria mucosa C102 |
| Dyella sp. SG562 | Lysobacter antibioticus | Neisseria sp. HMSC056A03 |
| Dyella sp. SG609 | Lysobacter capsici | Neisseria sp. HMSC064D07 |
| Dyella terrae | Lysobacter capsici AZ78 | Neisseria sp. HMSC066H01 |
| Dyella thiooxydans | Lysobacter enzymogenes | Neisseria sp. HMSC069H12 |
| Frateuria defendens | Lysobacter gummosus | Neisseria sp. HMSC06F02 |
| Frateuria terrea | Lysobacter lacus | Neisseria sp. HMSC070A01 |
| Hydrogenophaga crassostreae | Lysobacter maris | Neisseria sp. HMSC071B12 |
| Hydrogenophaga flava | Lysobacter oculi | Neisseria sp. HMSC071C03 |
| Hydrogenophaga pseudoflava | Lysobacter profundi | Neisseria sp. HMSC073B07 |
| Hydrogenophaga sp. | Lysobacter pythonis | Neisseria sp. oral taxon 014 str. F0314 |
| Hydrogenophaga sp. A37 | Lysobacter silvestris | Oxalobacteraceae bacterium |
| Hydrogenophaga sp. H7 | Lysobacter silvisoli | Pseudomonas aeruginosa |
| Hydrogenophaga sp. PAMC20947 | Lysobacter sp. 17J7-1 | Pseudomonas avellanae |
|-----------------------------|----------------------|----------------------|
| Pseudomonas savastanoi     | Rhodanobacter sp. DHB23 | Stenotrophomonas sp. CC120222-04 |
| Pseudomonas syringae group | Rhodanobacter sp. K2T2 | Stenotrophomonas sp. CC22-02 |
| genomosp. 3                 |                      |                      |
| Pseudomonas syringae pv. avellanae str. | Rhodanobacter sp. L36 | Stenotrophomonas sp. CF319 |
| ISPaVe013                  |                      |                      |
| Pseudoxanthomonas composti | Rhodanobacter sp. MP1X3 | Stenotrophomonas sp. HMWF023 |
| Pseudoxanthomonas dokdonensis | Rhodanobacter sp. OK091 | Stenotrophomonas sp. JAI102 |
| Pseudoxanthomonas gev      | Rhodanobacter sp. Root480 | Stenotrophomonas sp. LMG 10879 |
| Pseudoxanthomonas sacheonensis | Rhodanobacter sp. Root627 | Stenotrophomonas sp. MYb57 |
| Pseudoxanthomonas sp. CF125 | Stenotrophomonas acidaminiphila | Stenotrophomonas sp. NA06056 |
| Pseudoxanthomonas sp. GM95 | Stenotrophomonas chelatiphaga | Stenotrophomonas sp. Pemsol |
| Pseudoxanthomonas sp. GSS15 | Stenotrophomonas cyclobalanopsidis | Stenotrophomonas sp. pho |
| Pseudoxanthomonas sp. KAs_5_3 | Stenotrophomonas daejeonensis | Stenotrophomonas sp. RIT309 |
| Pseudoxanthomonas sp. NML130738 | Stenotrophomonas ginsengisoli | Stenotrophomonas sp. SAM-B |
| Pseudoxanthomonas sp. NML130969 | Stenotrophomonas indicatrix | Stenotrophomonas sp. SAU14A_NAIMI4_5 |
| Pseudoxanthomonas sp. NML140781 | Stenotrophomonas koreensis | Stenotrophomonas sp. SbOxS2 |
| Pseudoxanthomonas sp. NML160639 | Stenotrophomonas lactitubi | Stenotrophomonas sp. SPM |
| Pseudoxanthomonas sp. NML170316 | Stenotrophomonas maltophilia | Stenotrophomonas sp. TEPEL |
| Pseudoxanthomonas sp. NML171107 | Stenotrophomonas maltophilia 5BA-I-2 | Stenotrophomonas sp. VV52 |
|-------------------------------|-----------------------------------|--------------------------|
| Pseudoxanthomonas sp. NML171200 | Stenotrophomonas maltophilia Ab55555 | Stenotrophomonas sp. W1S232 |
| Pseudoxanthomonas sp. NML171202 | Stenotrophomonas maltophilia AU12-09 | Stenotrophomonas sp. WZN-1 |
| Pseudoxanthomonas sp. NML171590 | Stenotrophomonas maltophilia D457 | Stenotrophomonas sp. Y |
| Pseudoxanthomonas sp. NML171591 | Stenotrophomonas maltophilia EPM1 | Stenotrophomonas sp. YAU14A_MKIMI4_1 |
| Pseudoxanthomonas sp. NML180370 | Stenotrophomonas maltophilia JV3 | Stenotrophomonas sp. YAU14D1_LEIMI4_1 |
| Pseudoxanthomonas sp. NML180594 | Stenotrophomonas maltophilia K279a | Stenotrophomonas sp. YR399 |
| Pseudoxanthomonas sp. X-1 | Stenotrophomonas maltophilia M30 | Stenotrophomonas sp. ZAC14D2_NAIMI4_6 |
| Pseudoxanthomonas spadix | Stenotrophomonas maltophilia MF89 | Stenotrophomonas sp. ZAC14D2_NAIMI4_7 |
| Pseudoxanthomonas suwonensis | Stenotrophomonas maltophilia R551-3 | Streptomyces sp. SID10244 |
| Pseudoxanthomonas wuyuanensis | Stenotrophomonas maltophilia RA8 | Thermomonas fusca |
| Pseudoxanthomonas yeongjuensis | Stenotrophomonas maltophilia WJ66 | Variovorax paradoxus |
| Raoultella sp. 18102 | Stenotrophomonas pavanii | Variovorax sp. DXTD-1 |
| Rhodanobacter fulvus Jip2 | Stenotrophomonas pictorum | Variovorax sp. Root318D1 |
| Rhodanobacter glycini | Stenotrophomonas pictorum JCM 9942 | Variovorax sp. Sphag1AA |
| Rhodanobacter panaciterrae | Stenotrophomonas rhizophila | Variovorax sp. Sphag1J |
| Rhodanobacter sp. 67-28 | Stenotrophomonas sp. 278 | Vulcaniibacterium gelatinicum |
|------------------------|--------------------------|-------------------------------|
| Rhodanobacter sp. 7MK24 | Stenotrophomonas sp. 92mfcol6.1 | Vulcaniibacterium thermophilum |
| Rhodanobacter sp. A1T4 | Stenotrophomonas sp. AG209 | Xanthomonadaeae bacterium |
| Rhodanobacter sp. ANJX3 | Stenotrophomonas sp. ASS1 | Xanthomonadales bacterium 13-68-4 |
| Rhodanobacter sp. C01 | Stenotrophomonas sp. BIIR7 | Xanthomonadales bacterium 14-68-21 |
| Rhodanobacter sp. C06 | Stenotrophomonas sp. Br8 | Xanthomonadales bacterium 15-68-25 |
| Xanthomonadales bacterium | Xanthomonas citri pv. citri str. 306 | Xanthomonas sp. 3498 |
| Xanthomonadales bacterium | Xanthomonas citri pv. fuscans | Xanthomonas sp. 60 |
| Xanthomonas albilineans | Xanthomonas citri pv. glycines str. 8ra | Xanthomonas sp. CFBP 7698 |
| Xanthomonas arboricola pv. arracaciae | Xanthomonas citri pv. malvacearum | Xanthomonas sp. CFBP 7912 |
| Xanthomonas arboricola pv. celebensis | Xanthomonas citri pv. mangiferaeindicae LMG 941 | Xanthomonas sp. CPBF 426 |
| Xanthomonas arboricola pv. corylina | Xanthomonas citri pv. phaseoli var. fuscans | Xanthomonas sp. F1 |
| Xanthomonas arboricola pv. fragariae | Xanthomonas citri pv. vignicola | Xanthomonas sp. F14 |
| Xanthomonas arboricola pv. guizotiae | Xanthomonas citri subsp. citri A306 | Xanthomonas sp. F4 |
| Xanthomonas arboricola pv. juglandis | Xanthomonas citri subsp. citri Aw12879 | Xanthomonas sp. GPE 39 |
| Xanthomonas arboricola pv. populi | Xanthomonas codiaeai | Xanthomonas sp. GW |
| Xanthomonas arboricola pv. pruni MAFF 301420 | Xanthomonas cucurbitae | Xanthomonas sp. ISO98C4 |
|---------------------------------------------|------------------------|------------------------|
| Xanthomonas arboricola pv. pruni MAFF 301427 | Xanthomonas cynarae    | Xanthomonas sp. JAI131  |
| Xanthomonas arboricola pv. pruni str. MAFF 311562 | Xanthomonas dyei      | Xanthomonas sp. Leaf131 |
| Xanthomonas axonopodis pv. begoniae          | Xanthomonas euroxanthea| Xanthomonas sp. Leaf148 |
| Xanthomonas axonopodis pv. citrumeloi F1     | Xanthomonas euvesicatoria | Xanthomonas sp. LMG 12459 |
| Xanthomonas axonopodis pv. eucalyptorum      | Xanthomonas euvesicatoria pv. citrumeononis | Xanthomonas sp. LMG 12460 |
| Xanthomonas axonopodis pv. khayae            | Xanthomonas floridensis | Xanthomonas sp. LMG 12461 |
| Xanthomonas axonopodis pv. manihotis str. CIO151 | Xanthomonas fragariae LMG 25863 | Xanthomonas sp. LMG 12462 |
| Xanthomonas axonopodis pv. melhusii          | Xanthomonas gardneri   | Xanthomonas sp. LMG 8989 |
| Xanthomonas axonopodis pv. vasculorum        | Xanthomonas gardneri ATCC 19865 | Xanthomonas sp. LMG 8992 |
| Xanthomonas axonopodis Xac29-1               | Xanthomonas hortorum   | Xanthomonas sp. LMG 8993 |
| Xanthomonas campestris pv. arecae            | Xanthomonas hortorum pv. carotae str. M081 | Xanthomonas sp. LMG 9002 |
| Xanthomonas campestris pv. azadirachtae      | Xanthomonas hortorum pv. cynarae | Xanthomonas sp. MUS 060 |
| Xanthomonas campestris pv. badrii            | Xanthomonas hortorum pv. gardneri | Xanthomonas sp. NCPPB 1128 |
| Xanthomonas campestris pv. campestris str. 8004 | Xanthomonas hortorum pv. hederae | Xanthomonas sp. Sa3BUA13 |
| Xanthomonas campestris pv. campestris str. ATCC 33913 | Xanthomonas hortorum pv. pelargonii | Xanthomonas sp. SHU 166 |
| Xanthomonas campestris pv. campestris str. B100 | Xanthomonas hortorum pv. taraxaci | Xanthomonas sp. SHU 199 |
| Xanthomonas campestris pv. durantae | Xanthomonas hortorum pv. vitians | Xanthomonas sp. SI |
| Xanthomonas campestris pv. musacearum NCPPB 2251 | Xanthomonas hyacinthi | Xanthomonas sp. SS |
| Xanthomonas campestris pv. musacearum NCPPB 4379 | Xanthomonas hyacinthi DSM 19077 | Xanthomonas theicola |
| Xanthomonas campestris pv. musacearum NCPPB 4380 | Xanthomonas melonis | Xanthomonas translucens pv. arrhenatheri |
| Xanthomonas campestris pv. musacearum NCPPB 4384 | Xanthomonas nasturtii | Xanthomonas translucens pv. arrhenatheri LMG 727 |
| Xanthomonas campestris pv. raphani 756C | Xanthomonas perforans | Xanthomonas translucens pv. phlei |
| Xanthomonas campestris pv. vesicatoria str. 85-10 | Xanthomonas phaseoli | Xanthomonas translucens pv. poae |
| Xanthomonas campestris pv. vitiscarnosae | Xanthomonas phaseoli pv. dieffenbachiae | Xanthomonas translucens pv. translucens DSM 18974 |
| Xanthomonas cannabis pv. cannabis | Xanthomonas phaseoli pv. manihotis | Xanthomonas vasicola |
| Xanthomonas cannabis pv. phaseoli | Xanthomonas phaseoli pv. phaseoli | Xanthomonas vasicola pv. vasicola |
| Xanthomonas cassavae | Xanthomonas pisi | Xanthomonas vasicola pv. vasicola NCPPB 206 |
| Xanthomonas citri pv. aurantifolii | Xanthomonas prunicola | Xanthomonas vasicola pv. vasicola NCPPB 890 |
| Xanthomonas citri pv. citri | Xanthomonas sacchari | Xanthomonas vesicatoria ATCC 35937 |
Genomes listed code for an X-T4SS and putative X-Tfes based in the presence of an XVIPCD domain and VirB7 subunits with a C-terminal N0 domain. Hits were identified using the PSI-BLAST algorithm in the non-redundant protein database (GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects).
Table S5. Complex dissociation constants (K_D) calculated from ITC experiments.

| Protein pairs in titration                                      | K_D +/- sd (µM) |
|-----------------------------------------------------------------|-----------------|
| XAC2609-VirD4AAD                                                | 0.4 +/- 0.1     |
| XAC2609_XVIPCD-VirD4AAD                                         | 0.5 +/- 0.1     |
| XAC2609_XVIPCDΔ20-VirD4AAD                                      | 0.5 +/- 0.1     |
| XAC2609_XVIPCDH315A P316A D317A-VirD4AAD                         | 1.2 +/- 0.6     |
| XAC2609_XVIPCDG336W-VirD4AAD                                    | 0.5 +/- 0.3     |
| XAC2609_XVIPCDD363A-VirD4AAD                                    | 29.3 +/- 6.6    |
| XAC2609_XVIPCDF375A V377A-VirD4AAD                              | †ND             |
| XAC2609_XVIPCDD383A P384A-VirD4AAD                              | 28.1 +/- 25.0   |
| XAC2609_XVIPCDA396M-VirD4AAD                                    | 0.7 +/- 0.3     |
| XAC2609_XVIPCDP400A-VirD4AAD                                    | 0.9 +/- 0.6     |
| XAC2609_XVIPCDΔ6-VirD4AAD                                       | 0.6 +/- 0.1     |
| XAC2609_XVIPCDQ(397-431)A-VirD4AAD                              | 0.6 +/- 0.1     |
| XAC2609(392-431)-VirD4AAD                                       | †ND             |
| XAC2609(1-306)-VirD4AAD                                         | †ND             |
| PcfF-VirD4AAD                                                   | †ND             |
| PcfG-VirD4AAD                                                   | †ND             |
| VirD2-VirD4AAD                                                   | †ND             |
| XAC2609_XVIPCD-VirD4(487-557)                                   | †ND             |

K_D values are the average values from two to four independent experiments using a 1:1 binding model. sd, standard deviation.

†No interaction was measured in experiments.
Table S6. Oligonucleotides used in this study

| Oligonucleotides | Description | Sequence 5' - 3' |
|------------------|-------------|----------------|
| F/vtrD4 AAD/NdeI | Forward primer used to amplify virD4 (197-355) and cloning into pET28a vector at NdeI restriction site. | CACCAGCATATGATCGCTGGAAATCCATTGAC |
| R/vtrD4 AAD/XhoI | Reverse primer used to amplify virD4 (197-355) and cloning into pET28a vector at XhoI restriction site. | ATCTGCTCGAGTTAGTCATCTTCTGGCAGG |
| 1F/pBRA_3633/NcoI/GA | Forward primer used to amplify XAC3633-XAC3634 and cloning into pBRA vector at NcoI restriction site. | GGTAGCGAGGAATTACCATATGTCGTCAGGAAGATTTCACTGGTCC |
| 1R/pBRA_3634/SalI/GA | Reverse primer used to amplify XAC3633-XAC3634 and cloning into pBRA vector at SalI restriction site. | CAAGCATAAAGGCTGAGATCGATCGGTCAATTCAACGAGTACGAGC |
| xac2609/F/H315AP316AD317A | Forward primer for mutagenesis of XAC2609 at the codons 315, 316, and 317. | TTGTGCTGATCCTCGCGCTGCGCAACGCGCATGCAATCAACG |
| xac2609/R/H315AP316AD317A | Reverse primer for mutagenesis of XAC2609 at the codons 315, 316, and 317. | GGTGTAGAGGCTGATCGCGCTGCGCTGACCGCAATCAG |
| xac2609/F/G336W | Forward primer for mutagenesis of XAC2609 at the codon 336. | TGTTGTCTGATCCGCGCGCTGCCGCCAACGCGATGTACAACG |
| xac2609/R/G336W | Reverse primer for mutagenesis of XAC2609 at the codon 336. | CGTTGTACATCGCGTTGGCGGCAGCGCGCGGATCAGACAACA |
| xac2609/F/D363A | Forward primer for mutagenesis of XAC2609 at the codon 363. | CTTGCGGTTGGCAAACCAGCCACGTTCACCCAG |
| xac2609/R/D363A | Reverse primer for mutagenesis of XAC2609 at the codon 363. | CTGGGTGAACGTGGCTGGTTTGCCAACCGCAAG |
| xac2609/F/F375A V377A | Forward primer for mutagenesis of XAC2609 at the codons 375 and 377. | TCGCCCTGCGCTGCGGAAGCCGTCGCCGCTCT |
| xac2609/R/F375A V377A | Reverse primer for mutagenesis of XAC2609 at the codons 375 and 377. | AGAGCGGCGACGGTCTTGGCGACGCGAGGCG |
| xac2609/F/D383AP384A | Forward primer for mutagenesis of XAC2609 at the codons 383 and 384. | CGCTGCATTGCACCGCGCGTGACGTCGACG |
| xac2609/R/D383AP384A | Reverse primer for mutagenesis of XAC2609 at the codons 383 and 384. | GCCGAGCTGACCGCGCGGCAAATGCAACG |
| xac2609/F/A396M | Forward primer for mutagenesis of XAC2609 at the codon 396. | TCCAAAGGCGTATTCTGACATTATGATCGATCCAAACAAAC |
| xac2609/R/A396M | Reverse primer for mutagenesis of XAC2609 at the codon 396. | GCTTTTTTGTTGTGATCGCATAATTGCGAATATCGCGGGTTGGA |
| xac2609/F/P400A | Forward primer for mutagenesis of XAC2609 at the codon 400. | CTGCTGTTTTTCAACGCCGTGATTCTGCGGTGAG |
| xac2609/R/P400A | Reverse primer for mutagenesis of XAC2609 at the codon 400. | TCAAGCGGAGATCGGCGGTTGAAAAACAGCAG |
| xac2609/F/S412_Stop Codon | Forward primer for mutagenesis of XAC2609 at the codon 412. | TTGCTTGCTACGCATTTCCTCGCGCGCTTGG |
| xac2609/F/S412_Stop Codon | Reverse primer for mutagenesis of XAC2609 at the codon 412. | AGCAAGCGGCGACGGTGGAAATAGCAGCGTCAAG |
| xac3634/F/P514AD515A | Forward primer for mutagenesis of XAC3634 at the codons 514 and 515. | GGAGAAGAAAACACCGCGCACCCACCGCGTGTTC |
| xac3634/R/P514AD515A | Reverse primer for mutagenesis of XAC3634 at the codon 514 and 515. | GAACAGCGGCGGTGGCGCGGTGGTTTTCTCC |
| xac3634/F/D560A | Forward primer for mutagenesis of XAC3634 at the codon 560. | GCAATGCAACCAGCTCGCCATGCGTGGCCGGAAAG |
| xac3634/R/D560A | Reverse primer for mutagenesis of XAC3634 at the codon 560. | CTTTGGCACCACCGATCGCGGTGGTGCCATGC |
| xac3634/F/F572AV574A | Forward primer for mutagenesis of XAC3634 at the codon 572 and 574. | GGTGCGTACCCCTGCGCGCGACGCGATCGCGTC GCCAATC |
| xac3634/R/F572AV574A | Reverse primer for mutagenesis of XAC3634 at the codon 572 and 574. | GAGCGGGCCCAACGGTGGCGCACCCACCGCGCTGTTC |

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Forward primer for mutagenesis of XAC3634 at the codon 581.
GGTCAGCCAACCGCTGCGGCGCATCAGCG

Reverse primer for mutagenesis of XAC3634 at the codon 581.
CGCTGCGGAAACGTGGCGCTGGAG

Forward primer for mutagenesis of XAC3634 at the codon 597.
CTCCAGCGAGGTTCGCGACGGG

Reverse primer for mutagenesis of XAC3634 at the codon 597.
CGGCCGCAGTCGTACGTTGGCGAAAC

Forward primer for mutagenesis of XAC3634 at the codon 622.
AAGATCAGCAGGCAATACGTTAGGCCAGCGG

Reverse primer for mutagenesis of XAC3634 at the codon 622.
GGGCTGGAGGC TGCCCTGTGCCGGCGCAG

Forward primer used to amplify xac2609 (1-308) with the reverse primer R_2609NT(308) and the plasmid pBRA-XAC2609 as template. This amplicon was used with the PCR product of F_XVIPCD(302) and R_2609XVIPCD(431)pBRA primers and the opened plasmid pBRA at Ncol/Sal cleavage site for the Gibson assembly reaction step to produce the vector pBRA-XAC2609Q(397-431)A.
GGCTAGCAGGAGGAATTCACCATGGGCGATGGACGTGGTCGGAG

Reverse primer used to amplify xac2609(1-308) with the forward primer F_pBRA_2609NT(1) and the plasmid pBRA-XAC2609 as template.
GGGCTGGAGGC TGCCCTGTGCCGGCGCAG

Forward primer to amplify XAC2609Q(397-431)A(302-431) with the pair primer R_2609XVIPCD(431)pBRA and the plasmid pET28a-XAC2609HIS-306-431Q(397-431)A as template.
GCACAGGCAGCCTCCAGCCCTTTGTTGTC

Reverse primer to amplify XAC2609Q(397-431)A with the pair primer F_XVIPCD(302) and pET28a-XAC2609HIS-306-431Q(397-431)A as template.
CAAGCATAAAGCCCTGACGGTCGACTTACATGGACATTGAGCGC

Forward primer used to amplify XAC3634(1-513) and cloning into pET28a vector at NdeI restriction site.
TTCTTGTCATATGCGCAGCAACTATTCACG

Reverse primer used to amplify XAC3634(1-513) and cloning into pET28a vector at BamHI restriction site.
TCAGGATCCTTGGTGGTTGGTTTGAGCA

Forward primer used to amplify XAC3634(40-300) and cloning into pET28a vector at NdeI restriction site.
GCCCATATGAAAACCCTCAGAAAAGGATGCA

Reverse primer used to amplify XAC3634(40-300) and cloning into pET28a vector at BamHI restriction site.
TCAGGATCCTCAAGGGGTGCGGCAAATAC

Annealing at 877 bp upstream the starting codon of XAC2884.
TCATTCCGATCCACCTCAAGGCAACCCCGAT

PCR pair primer with R1 ∆2884 Xhol to produce the amplicon used in the construction of the pNPTS\(\Delta\)XAC2885/XAC2884 plasmid.

Annealing at the codon 36 of XAC2884. PCR pair primer with F1 ∆2884 Xhol to produce the amplicon used in the construction of the pNPTS\(\Delta\)XAC2885/XAC2884 plasmid.

Annealing at the codon 304 of XAC2885. PCR pair primer with R2 ∆2884 EcoRI to produce the amplicon used in the construction of the pNPTS\(\Delta\)XAC2885/XAC2884 plasmid.
**R2 ∆2884 ∆2885 EcoRI**

Annealing at 1003 bp downstream of the termination codon of XAC2885. PCR pair primer with F2 ∆2884 ∆2885 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC2885/XAC2884 plasmid.

```
TATCAGGAATTCCGAAGATCGATGCACGCAG
```

**F1 ∆0573 ∆0574 BamHI**

Annealing at 1015 bp upstream the starting codon of XAC0573. PCR pair primer with R1 ∆0573 ∆0574 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC0573/XAC0574 plasmid.

```
TCATTCGGATCCCTGCGCTATGGGTCAGAAAGC
```

**R1 ∆0573 ∆0574 XhoI**

Annealing at the starting codon of XAC0573. PCR pair primer with F1 ∆0573 ∆0574 BamHI to produce the amplicon used in the construction of the pNPTSΔXAC0573/XAC0574 plasmid.

```
TTCATGCTCGAGCAATCTAGTCCGCTCGCGTTC
```

**F2 ∆0573 ∆0574 XhoI**

Annealing at the codon 438 of the XAC0574. PCR pair primer with R2 ∆0573 ∆0574 EcoRI to produce the amplicon used in the construction of the pNPTSΔXAC0573/XAC0574 plasmid.

```
AATGTCCTCGAGCACCTGGGCTAAGGGGAGC
```

**R2 ∆0573 ∆0574 EcoRI**

Annealing at 1024 bp downstream of the termination codon of XAC0574. PCR pair primer with F2 ∆0573 ∆0574 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC0573/XAC0574 plasmid.

```
TATCAGGAATTCCGTTGGGATACACCGCCTTGG
```

**F1 ∆0096 ∆0097 NheI**

Annealing at 1033 bp upstream the starting codon of XAC0097. PCR pair primer with R1 ∆0096 ∆0097 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC0097/XAC0096 plasmid.

```
TATCAGGGCTAGCATCGGAGCTGTGGTTGATG
```

**R1 ∆0096 ∆0097 XhoI**

Annealing at 10 pb downstream the starting codon of XAC0097. PCR pair primer with F1 ∆0096 ∆0097 NheI to produce the amplicon used in the construction of the pNPTSΔXAC0097/XAC0096 plasmid.

```
TTCATGCTCGAGGAAGCGCATTAGCAGATCC
```

**F2 ∆0096 ∆0097 XhoI**

Annealing starts at the codon 628 of the XAC0096. PCR pair primer with R2 ∆0096 ∆0097 NheI to produce the amplicon used in the construction of the pNPTSΔXAC0097/XAC0096 plasmid.

```
AATGTCCTCGAGCTGTCCCGCTAGATCGCAC
```

**R2 ∆0096 ∆0097 NheI**

Annealing starts 1005 bp downstream the termination codon of XAC0096. PCR pair primer with F2 ∆0096 ∆0097 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC0097/XAC0096 plasmid.

```
TCATCGCTAGCGCGCAGCATCATGTTCTCGAC
```

**F1 ∆3633 ∆3634 BamHI**

Annealing at 860 bp upstream the starting codon of XAC3633. PCR pair primer with R1 ∆3633 ∆3634 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC3633/XAC3632 plasmid.

```
TATCAGGGATTCCGTTGGCCCTCTCCATCAACG
```
amplicon used in the construction of the pNPTSΔXAC3633/XAC3634 plasmid.

**R1 Δ3633 Δ3634 XhoI**
Annealing at 162 pb downstream the starting codon of XAC3633. PCR pair primer with F1 Δ3633 Δ3634 BamHI to produce the amplicon used in the construction of the pNPTSΔXAC3633/XAC3634 plasmid.

TCATTCCCTCGAGCTGGGTGGTGCCTGCAATG

**F2 Δ3633 Δ3634 XhoI**
Annealing at the codon 628 of the XAC3634. PCR pair primer with R2 Δ3633 Δ3634 BamHI to produce the amplicon used in the construction of the pNPTSΔXAC3633/XAC3634 plasmid.

TTCATGCTCGAGACGGTATGGCTGATTGTC

**R2 Δ3633 Δ3634 BamHI**
Annealing at 1023 bp downstream the termination codon of XAC3634. PCR pair primer with F2 Δ3633 Δ3634 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC3633/XAC3634 plasmid.

AATGTCGGATCCGATGGCAGCTGAGTGC

**F1 Δ1917 Δ1918 BamHI**
Annealing starts 9 pb downstream of the starting codon of XAC1917. PCR pair primer with R1 Δ1917 Δ1918 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC1917/XAC1918 plasmid.

TTCATGCTCGAGGTATCCACGGGAGCCCAC

**R1 Δ1917 Δ1918 XhoI**
Annealing at the codon 602 of the XAC1918. PCR pair primer with R2 Δ1917 Δ1918 BamHI to produce the amplicon used in the construction of the pNPTSΔXAC1917/XAC1918 plasmid.

AATGTCCTCGAGCAACCAACCAGGTGTGAG

**F2 Δ1917 Δ1918 XhoI**
Annealing at 954 bp downstream of the termination codon of XAC1918. PCR pair primer with F2 Δ1917 Δ1918 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC1917/XAC1918 plasmid.

TATCAGGGATCTGGGCTATCACCACAAG

**R2 Δ1917 Δ1918 BamHI**
Annealing at 970 bp upstream the starting codon of XAC0467. PCR pair primer with F1 Δ0466 Δ0467 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC0467/XAC0466 plasmid.

TATCAGGGATCTGGGCTATCACCACAAG

**F1 Δ0466 Δ0467 NheI**
Annealing at 42 pb downstream of the starting codon of XAC0467. PCR pair primer with F1 Δ0466 Δ0467 NheI to produce the amplicon used in the construction of the pNPTSΔXAC0467/XAC0466 plasmid.

TTCATGCTCGAGCTGACGACTGGTCG

**R1 Δ0466 Δ0467 XhoI**
Annealing at 42 pb downstream of the starting codon of XAC0467. PCR pair primer with F1 Δ0466 Δ0467 XhoI to produce the amplicon used in the construction of the pNPTSΔXAC0467/XAC0466 plasmid.
Annealing at the stop codon of the XAC0466. PCR pair primer with R2 ∆0466 ∆0467 NheI to produce the amplicon used in the construction of the pNPTS ∆XAC0467/XAC0466 plasmid.

Annealing starts at 1094 bp downstream of the stop codon of XAC0466. PCR pair primer with F2 ∆0466 ∆0467 XhoI to produce the amplicon used in the construction of the pNPTS ∆XAC0467/XAC0466 plasmid.

Annealing at 870 bp upstream of the starting codon of XAC4262. PCR pair primer with R1 XAC ∆XAC4264/3/2 to produce the amplicon used in the construction of the pNPTS ∆XAC4264/4263/422 plasmid.

Annealing at the codon 302 of XAC4264. PCR pair primer R2 XAC Delta XAC4264/3/2 to produce the amplicon used in the construction of the pNPTS ∆XAC4264/4263/422 plasmid.

Annealing starts at 911 bp downstream of the stop codon of XAC4264. PCR pair primer with F2 XAC Delta XAC4264/3/2 to construct the insert for the pNPTS ∆XAC4264/4263/422 plasmid.

Annealing 995 bp upstream from the starting codon of XAC2609. PCR pair primer with R1 Transl msfGFP/Xac2609 to produce the amplicon used in the construction of the pNTPS-∆2609::msfGFP.

Annealing starts at 5 bp downstream of the starting codon of XAC2609 (italic). PCR pair primer with F1 Transl msfGFP/Xac2609 to produce the PCR product was used in the construction of the pNTPS-∆2609::msfGFP.

Annealing starts at 5 pb downstream of the starting codon of XAC2609. PCR pair primer with CW31_msfGFP R using the plasmid pDHL1029 (6) as template. PCR product was used in the construction of the pNTPS-∆2609::msfGFP.

Forward primer to amplify msfGFP starting at 5 pb downstream of the starting codon. PCR pair primer with CW31_msfGFP R using the plasmid pDHL1029 (6) as template. PCR product was used in the construction of the pNTPS-∆2609::msfGFP.
Reverse primer to amplify msfGFP 1 pb upstream from the stop codon. PCR pair primer with CW30_msfGFP F using the plasmid pDHL1029 (6) as template. PCR product was used in the construction of the pNTPS-∆2609::msfGFP.

TTTGTAGAGTTCCATCCATGC

Forward primer used to amplify the downstream region of the XAC2609, annealing starts at the stop codon of the XAC2609 (italic). PCR pair primer with R2 Transl GFP/Xac2609. PCR product was used in the construction of the pNTPS-∆2609::msfGFP.

ATCACGCACGGCATGGATGACCTCTAACAATAAAGTGGCGTGTTGTC

Reverse primer used to amplify the downstream region of XAC2609, annealing starts at 983 bp after the stop codon of XAC2609. PCR pair primer with F2 Transl msfGFP/Xac2609. PCR product was used in the construction of the pNTPS-∆2609::msfGFP.

F1pNPTSFup3267

Annealing 1000 bp from the starting codon of XAC3267. PCR pair primer with R1up3267 to build the plasmid pNPTS\_XAC3267/3266.

ATATGGATCCTAGTTGCCCGCTGCGCTACC

Annealing at 60 bp downstream of the starting codon of XAC3267. PCR pair primer with F1pNPTSFup3267 to build the plasmid pNPTS\_XAC3267/3266.

CTGCTGTGCATTTCACTGGGAACGTAAACTGGAAGAACTC

Annealing at the codon 845 of XAC3266. PCR pair primer with R2down3266pNPTS to build the plasmid pNPTS\_XAC3267/3266.

GTCCCCGATGAATTCACACAGCAGCAGACTGCACG

Annealing at 889 bp downstream of XAC3266 stop codon. PCR pair primer with F2down3266 to build the plasmid pNPTS\_XAC3267/3266.

ATATGGATCCTAGTTGCCCGCTGCGCTACC

F-Primer used for yeast two hybrid assays cloning VirD4 into the pOBD vector.

AADFTH

gacggaattcACGCATCGCTGGAATCCATTG

R-Primer used for yeast two hybrid assays cloning VirD4 into the pOBD vector.

ADR

taggtggatcgattgaggggcaaggccgccc

F-VirD4-83-Ndel

Forward primer to cloning VirD4 gene into pET28a vector.

R-VirD4-557HindI

Reverse primer to cloning VirD4 gene into pET28a vector.

F-VirD4-487-557 sumoGA

Forward primer for cloning VirD4(487-557) into pSUMO vector.

R-VirD4-487-557 sumoGA

Reverse primer for cloning VirD4(487-557) into pSUMO vector.

F-VirD4-83-Ndel GA

Forward primer to cloning VirD4 gene into pET11a vector.

R-VirD4-557-Ndel GA

Forward primer to cloning VirD4 gene into pET11a vector.
Table S7. Plasmids used for heterologous protein expression in *E. coli* BL21(DE3) strain and purification

| Plasmid | Expression condition (Temperature/Time) | Purification Steps [(first step)/(second step)] | Recombinant Proteins | Reference |
|---------|----------------------------------------|-------------------------------------------------|----------------------|-----------|
| pET28a-VirD4[HIS-197-355] | 18 °C / 16 hrs | Affinity chromatography/SEC | HIS-VirD4AAD/$^+$VirD4AAD | This work |
| *pET11a-VirD4*<sub>(197-355)</sub> | 18 °C / 16 hrs | Co-purified as prey for pull down assay by Affinity chromatography | VirD4<sub>AAD</sub> | This work |
| pET28a-VirD4[HIS-83-557] | 18 °C / 16 hrs | Affinity chromatography | VirD4<sub>(HIS-83-557)</sub> | This work |
| pET11a-VirD4<sub>(83-557)</sub> | 18 °C / 16 hrs | Co-purified as prey for pull down assay by Affinity chromatography | VirD4<sub>(83-557)</sub> | This work |
| pSUMO-VirD4[HIS-SUMO-487-557] | 18 °C / 16 hrs | Affinity chromatography | $^1$VirD4(487-557) | This work |
| pET11a-XAC2609<sub>311-431</sub> | 37 °C / 3 hrs | Anion-exchange chromatography/SEC and as prey for pull down assay by Affinity chromatography | XAC2609<sub>XVIPCD</sub> | (1) |
| pET11a-XAC2609<sub>-431</sub> | 37°C/ 3 hrs | Anion-exchange chromatography/SEC | XAC2609 | (2) |
| pET11a-XAC2609<sub>-306</sub> | 37°C/ 3 hrs | Anion-exchange chromatography/SEC | XAC2609(1-306) | (1) |
| *pET28a-XAC2609<sub>HIS-311-431</sub>*<sup>D317A</sup> | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609<sub>XVIPCD</sub>/"XAC2609<sub>XVIPCD</sub>" | This work |
| pET28a-XAC2609<sub>HIS-311-431</sub><sup>H315A P316A D317A</sup> | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609<sub>XVIPCD</sub>H315A P316A D317A | This work |
| pET28a-XAC2609<sub>HIS-311-431</sub><sup>G336W</sup> | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609<sub>XVIPCD</sub>G336W | This work |
| pET28a-XAC2609<sub>HIS-311-431</sub><sup>D363A</sup> | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609<sub>XVIPCD</sub>D363A | This work |
| Construct                  | Temperature | Duration | Expression Method | Fusion Tag | Modification          | Source |
|----------------------------|-------------|----------|-------------------|------------|-----------------------|--------|
| pET28a-XAC2609\_F375A\_V377A | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609\_XVIPCD\_F375A\_V377A | This work |
| pET28a-XAC2609\_D383A\_P384A | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609\_XVIPCD\_D383A\_P384A | This work |
| pET28a-XAC2609\_A396M | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609\_XVIPCD\_A396M | This work |
| pET28a-XAC2609\_P400A | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609\_XVIPCD\_P400A | This work |
| pET28a-XAC2609\_S426STer | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609\_XVIPCD\_S426STer | This work |
| pET28a-XAC2609\_Q(397-431)A | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609\_XVIPCD\_Q(397-431)A | This work |
| pET28a-XAC2609\_Δ2O | 18 °C / 16 hrs | Affinity chromatography | HIS-XAC2609\_XVIPCD\_Δ2O | This work |
| pET28a-XAC3633\_Q(40-300) | 18 °C / 16 hrs | Affinity chromatography | XAC3633\_Q(40-300) | This work |
| pPC2071 | 18 °C / 16 hrs | Affinity chromatography | His-VirD2 | (29) |
| pCY36 | 18 °C / 16 hrs | Affinity chromatography | pcfG-His | (33) |
| pCY33 | 18 °C / 16 hrs | Affinity chromatography | His6-pcfF | (33) |
| pET28a-XAC3634\_Δ20 | 37°C / 3 hrs | Affinity chromatography | XAC3634\_Δ20 | (33) |
| pET28a-XAC3634\_E35Q | 37°C / 3 hrs | Affinity chromatography | XAC3634\_E35Q | (33) |

% Named follow cleavage of the N-Terminal Histidine tail.

*pET11a-VirD4\_197-355 was produced by subcloning the insert Ndel/Xhol from pET28a-VirD4\_HIS-197-355 into a vector pET11a modified vector coding for Ndel/Xhol restriction sites.

†pET28a-XAC2609\_HIS-311-431 was produced by subcloning the insert from pET11a-XAC2609\_HIS-311-431 into vector pET28a using Ndel/BamHI cleavage sites.

‡1 ‡2The synthetic gene produced by GenScript, ‡1 all the glutamine codons between the residues 397-431 of xac2609 were replaced by alanine codons.

$1VirD4(487-557) is obtained after the HIS-SUMO-VirD4(487-557) cleavage with Ulp1 protease (4).
### Table S8. Plasmids used in *X. citri* strains and *E. coli* in interspecies competition assays.

| Plasmid                        | Recombinant Proteins produced | Recipient strain                |
|-------------------------------|-------------------------------|---------------------------------|
| pBRA-empty                    | --                            | *X. citri Δ7Δ2609-GFP*          |
| pBRA-empty                    | --                            | *X. citri ΔVirB7* (34)(1)       |
| pBRA-empty                    | --                            | *X. citri ΔVirD4* (1)           |
| pBRA-XAC2609 (1)              | XAC2609                       | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609 (1)              | XAC2609(1-306)                | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609G336W              | XAC2609 G336W                 | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609D363A             | XAC2609 D363A                 | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609F375A V377A       | XAC2609 F375A V377A           | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609D383A P384A       | XAC2609 D383A P384A           | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609A396M             | XAC2609 A396M                 | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609P400A             | XAC2609 P400A                 | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609S426Ter           | XAC2609 S426Ter               | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609Q(397-431)A       | XAC2609 Q(397-431)A           | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609R427A             | XAC2609 R427A                 | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC2609Δ20               | XAC2609 Δ20                   | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC3633-XAC3634          | XAC3633 + XAC3634             | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC3633-XAC3634P514A D515A | XAC3633 + XAC3634 P514A D515A | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC3633-XAC3634D560A     | XAC3633 + XAC3634 D560A       | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC3633-XAC3634F572A V574A | XAC3633 + XAC3634 F572A V574A | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC3633-XAC3634P597A     | XAC3633 + XAC3634 P597A       | *X. citri Δ7Δ2609-GFP*          |
| pBRA-XAC3633-XAC3634Q622Ter   | XAC3633 + XAC3634 Q622Ter     | *X. citri Δ7Δ2609-GFP*          |
| pBRA-VirB7 (1)                | VirB7                         | *X. citri ΔVirB7* (1)           |
| pBBRGFP (1)                   | GFP protein                   | *X. citri WT, X. citri ΔVir7   |
| pOBDAAD                       | VirD4AAD                      | Yeast                          |
| pBBR(1)RFP (reference 4)      | RFP protein                   | *E. coli BL21(DE3)RIL ArcticExpress* |
**Table S9. Construction of *X. citri* strain Δ7Δ2609-GFP and Δ8Δ2609-GFP strains by successive two-step allelic exchanges.**

| Plasmid pNPTSΔX-Tfi/X-Tfe | Recipient strain      | *X. citri* strain after recombination |
|---------------------------|-----------------------|---------------------------------------|
| pNPTSΔXAC2885/XAC2884     | *X. citri* wild type  | *X. citri* Δ1                         |
| pNPTSΔXAC0574/XAC0573     | *X. citri* Δ1         | *X. citri* Δ2                         |
| pNPTSΔXAC0097/XAC0096     | *X. citri* Δ2         | *X. citri* Δ3                         |
| pNPTSΔXAC3633/XAC3634     | *X. citri* Δ3         | *X. citri* Δ4                         |
| pNPTSΔXAC1918/XAC1917     | *X. citri* Δ4         | *X. citri* Δ5                         |
| pNPTSΔXAC0467/XAC0466     | *X. citri* Δ5         | *X. citri* Δ6                         |
| pNPTSΔXAC4264/XAC4263/XAC4262 | *X. citri* Δ6      | *X. citri* Δ7                         |
| pNTPS-Δ2609::msfGFP       | *X. citri* Δ7         | *X. citri* Δ7Δ2609-GFP                |
| pNPTS3266/3267           | *X. citri* Δ7Δ2609-GFP | *X. citri* Δ8Δ2609-GFP               |
Movie S1. *X. citri* Δ7Δ2609 vs *E. coli*. Time-lapse microscopy movies showing *X. citri* Δ7Δ2609-GFP (green) carrying the empty pBRa vector interacting with *E. coli* BL21(DE3)RIL ArcticExpress expressing RFP (red). Images were acquired every 10 min. Timestamps in hours:minutes. Scale bar 5 μm. No *E. coli* killing events were observed.

Movie S2. *X. citri* Δ7Δ2609 expressing X-TfeXAC2609 vs *E. coli*. Time-lapse microscopy movies showing *X. citri* Δ7Δ2609-GFP carrying pBRA-XAC2609 vector interacting with *E. coli* BL21(DE3)RIL ArcticExpress expressing RFP (red). Images were acquired every 10 min. Timestamps in hours:minutes. Scale bar 5 μm. *E. coli* killing events were only observed when in contact with *X. citri* Δ7Δ2609-GFP carrying pBRA-XAC2609.

Movie S3. *X. citri* Δ7Δ2609 expressing X-TfeXAC3634 vs *E. coli*. Time-lapse microscopy movies showing *X. citri* Δ7Δ2609-GFP carrying the pBRA-XAC3633-XAC3634 vector interacting with *E. coli* BL21(DE3)RIL ArcticExpress expressing RFP (red). Images were acquired every 10 min. Timestamps in hours:minutes. Scale bar 5 μm. *E. coli* killing events were only observed when in contact with *X. citri* Δ7Δ2609-GFP carrying pBRA-XAC3633-XAC3634.

Dataset S1. Sequence alignment of the XVIPCD N-terminal ααβββ region of 4756 XVIPCD sequences from X-Tfes identified shown in Figure 2A of the main manuscript. This file includes the NCBI reference code for each target identified in the protein databases. They are provided in PFAM format and can be conveniently visualized using Jalview software (https://www.jalview.org/).

Dataset S2. Sequences of the XVIPCD C-terminal tails shown in Figure 2B of the main manuscript. Sequences are ordered according to length and aligned from their C-terminii. This file includes the NCBI reference code for each target identified in the protein databases. They are provided in PFAM format and can be conveniently visualized using Jalview software (https://www.jalview.org/).
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