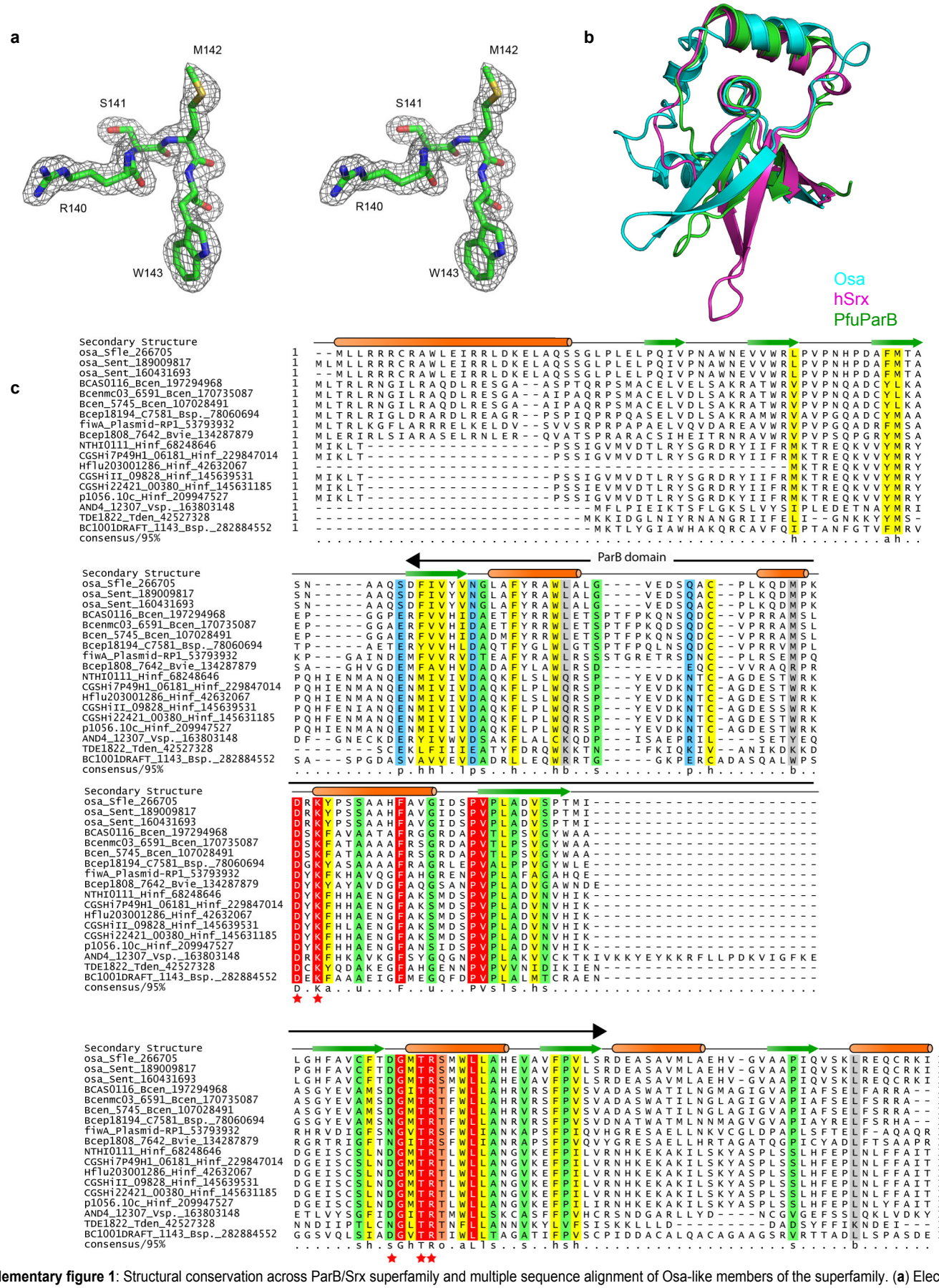
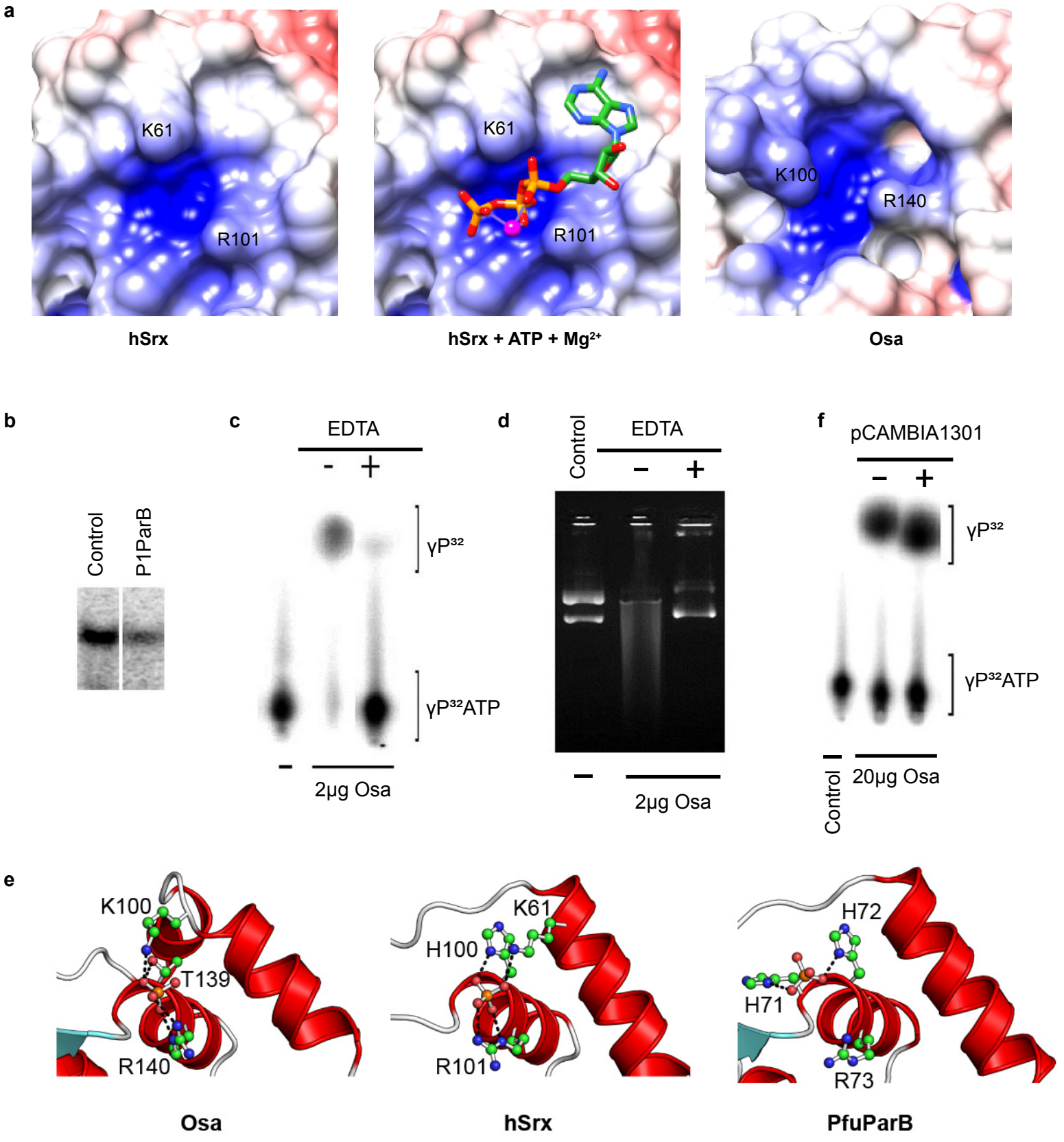
Supplementary Figure 1 (arockiasamy)

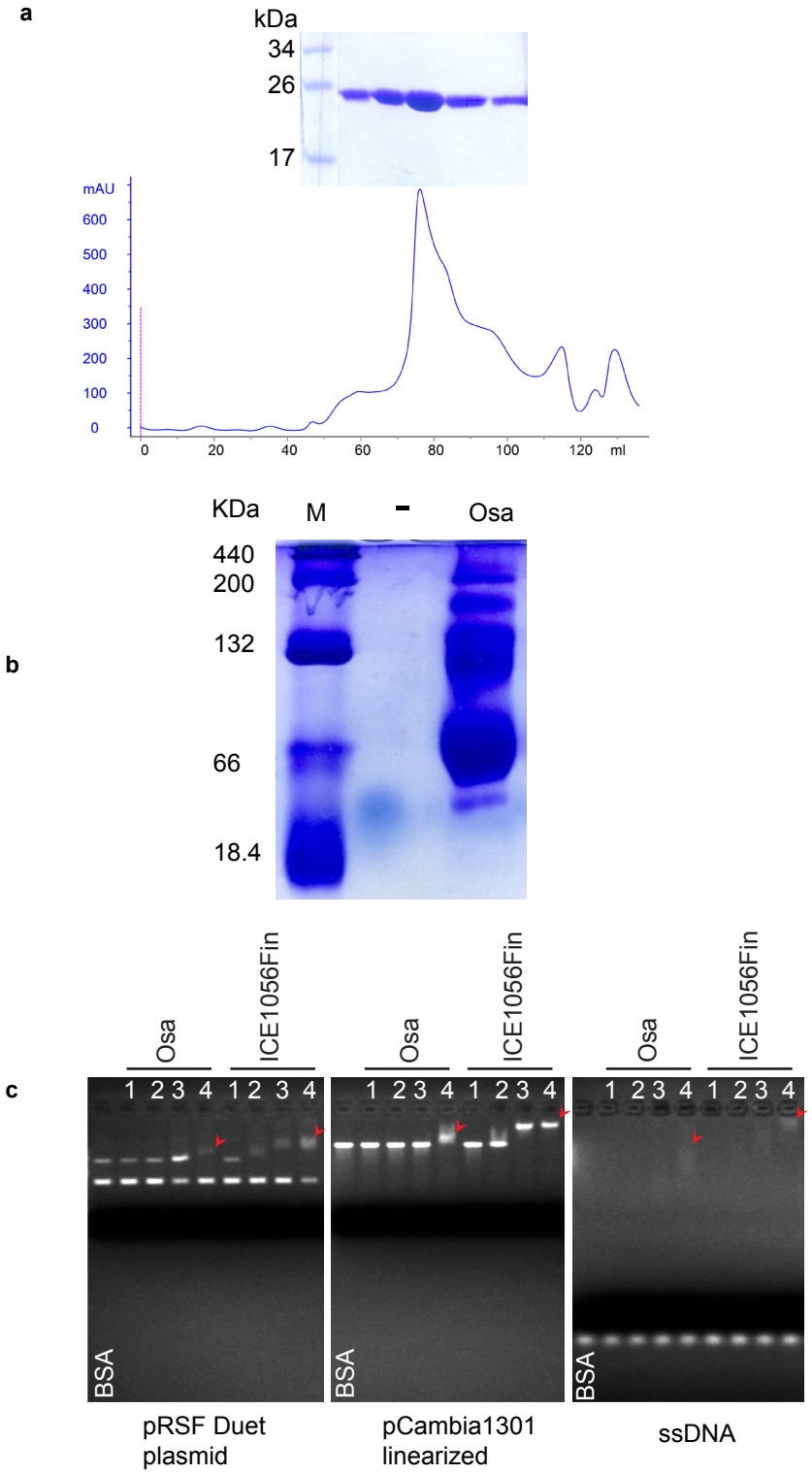


Supplementary figure 1: Structural conservation across ParB/Srx superfamily and multiple sequence alignment of Osa-like members of the superfamily. (**a**) Electron density map (2Fo-Fc) for a selected region of Osa contoured at 1.0 σ. (**b**) Structural superposition of ParB/Srx core region of Osa, hSrX and PfuParB. (**c**) Multiple sequence alignment of Osa-like fertility inhibition factors within the ParB/Srx superfamily showing conserved and predicted active site residues marked with red stars. Protein sequences are labeled by their gene names (where available),species abbreviations and Genbank index (GI) numbers. The alignment is colored at 95% consensus using the following scheme: : h, hydrophobic (ACFGHILMTVWY), l, aliphatic (ILAV) and a, aromatic (FYH) residues shaded yellow; p, polar (CDEHKNQRST) residues colored blue; s, small (ACDGNPSTV) and u, tiny (GAS) residues colored green, big residues (QRKEILMWYF) shaded grey; o, alcohol group (ST) containing residues shaded orange and absolutely conserved residues are shaded red. Species abbreviations are as follows: Bcen : *Burkholderia cenocepacia*; Blat : *Burkholderia lata*; Burk : *Burkholderia sp.*; Bvie : *Burkholderia vietnamiensis*; Hinf : *Haemophilus influenzae*; Sent : *Salmonella enterica*; Sfle : *Shigella flexneri*; Tden : *Treponema denticola*; Vsp. : *Vibrio sp.*

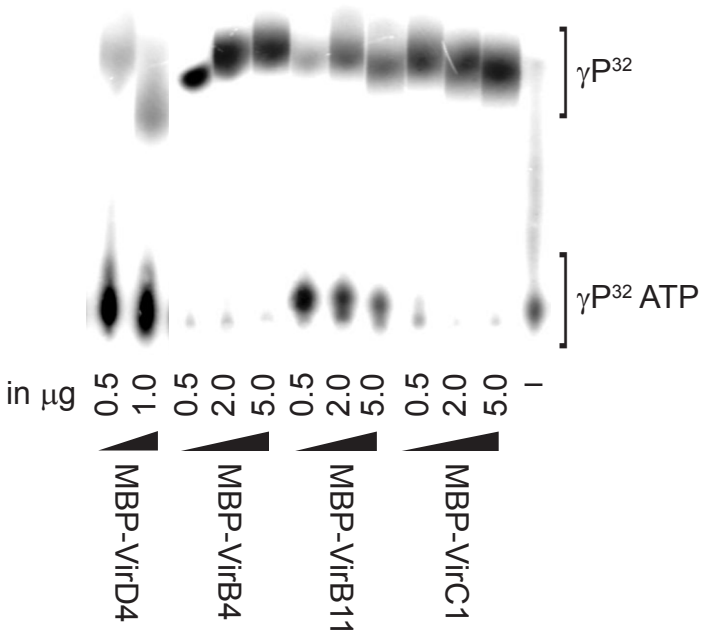


Supplementary figure 2: Comparison of active site of hSrx with the predicted functional site of Osa, and associated biochemical activities.(**a**) ATP binding pocket of hSrx without ATP, with bound ATP in the active site of hSrx (PDB:3cyi) is shown along with the predicted ATP binding pocket of Osa. (**b**) DNase activity of P1ParB using 90-mer ssDNA substrate. (**c**) ATPase and (**d**) DNase activity of Osa in the presence and absence of EDTA. (**e**) Conserved phosphate binding site in the crystal structures of ParB/Srx superfamily proteins are shown. (**f**) ATPase activity of Osa in absence or presence of 1 μg of plasmid pCAMBIA1301 in the reaction mix.

Supplementary Figure 3 (arockiasamy)



Supplementary figure 3: Multimerization is conserved across the ParB/Srx superfamily. (**a**) Gel filtration chromatogram showing multiple peaks containing pure Osa, which was purified to homogeneity even before loading onto Superdex-S200 column. Corresponding eluted fractions were run on SDS-PAGE (inlet). (**b**) Blue-Native PAGE of purified Osa shows multimerization in the absence of substrate DNA. (**c**) Gel-shift assay with Osa and Ice1056Fin showing supershifting of DNA seen with uncut plasmid (pRSF-Duet1) as non-specific DNA, linearized dsDNA (pCAMBIA1301) with T-DNA borders, and 90mer ssDNA with left and right borders substrates. The reaction carried out for 15 minutes on ice and ran on 1% and 2.5% Agarose gels for dsDNA and ssDNA, respectively. Samples loaded in lanes 1-4 had 0.5, 1, 2.5, 10 µg, respectively, of corresponding FIN proteins. The concentrations used were 50 and 500 ng for dsDNA and ssDNA, respectively.

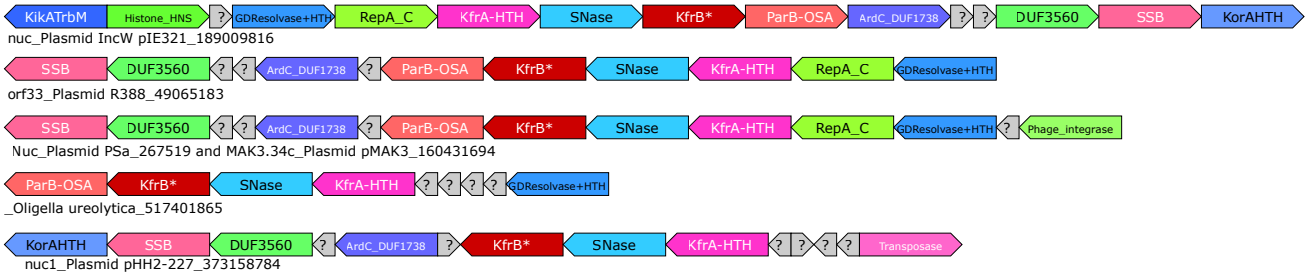


Supplementary figure 4: ATPase activity of purified VirB/D4 T4SS components; VirD4, VirB4, VirB11 and the relaxosome ATPase VirC1. [γ-P32]ATP was used as substrate in the assay and the reaction products were run on TLC.

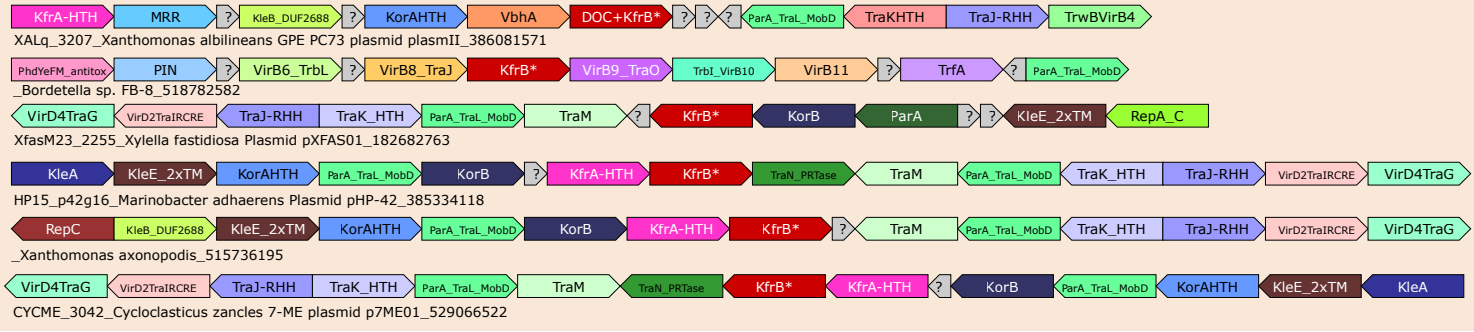
Supplementary Figure 5a (arockiasamy)

a

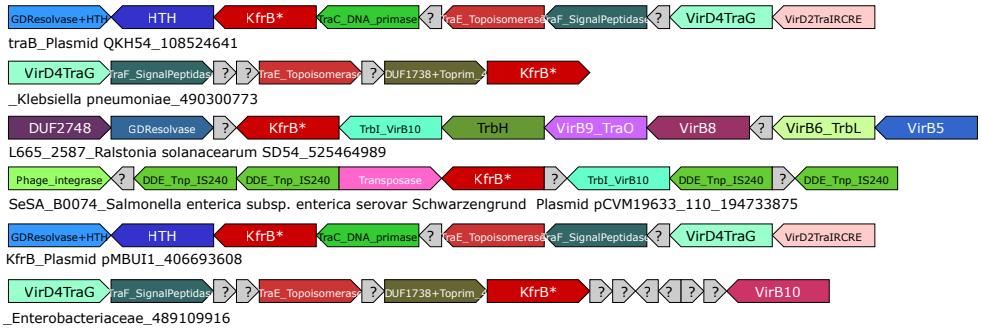
OSA-associated



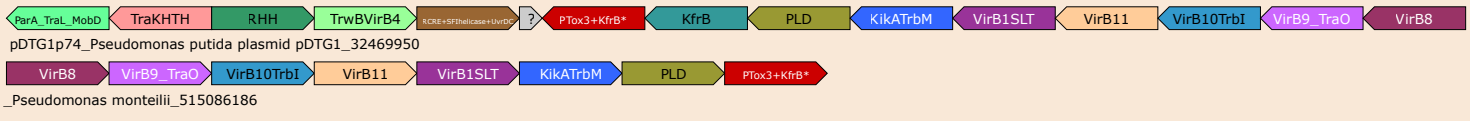
T4SS and Relaxase (RCRE) system associated



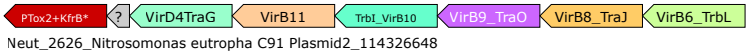
T4SS associated



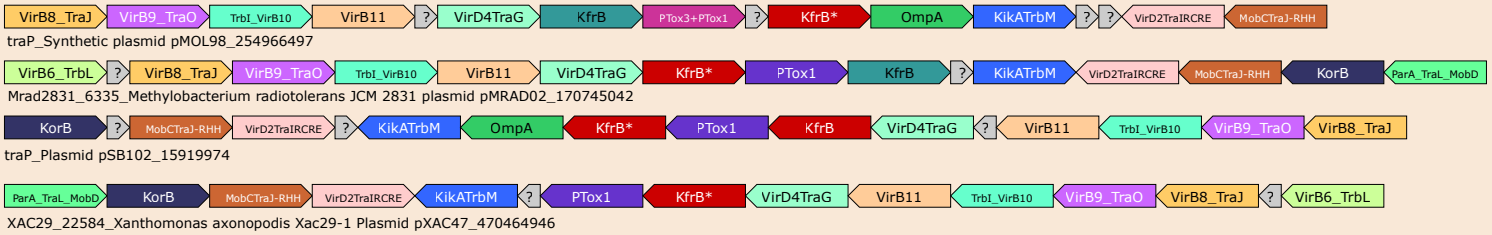
PTox3, T4SS and Relaxase (RCRE) associated



PTox2 and T4SS associated

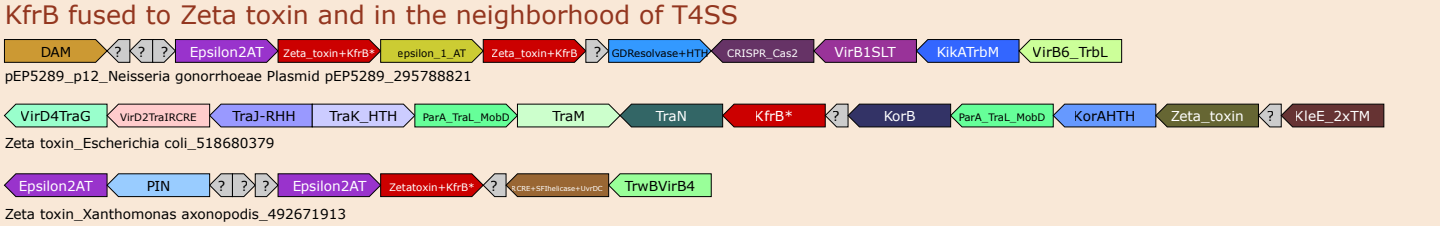


PTox1-associated or fused and T4SS and Mob associated



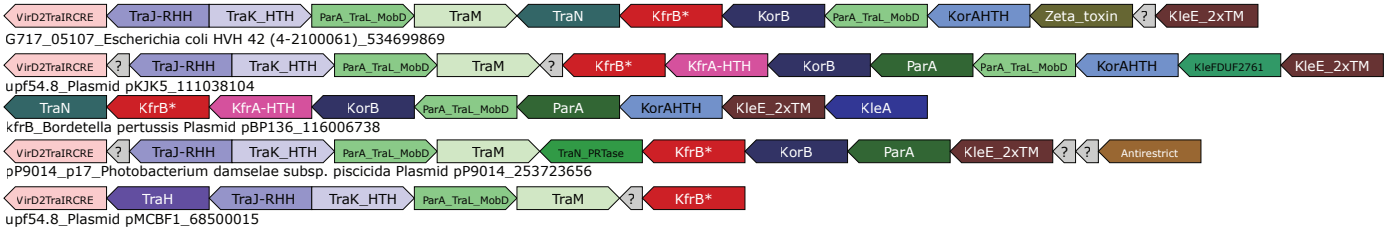
Fused to PTox1

KfrB fused to Zeta toxin and in the neighborhood of T4SS

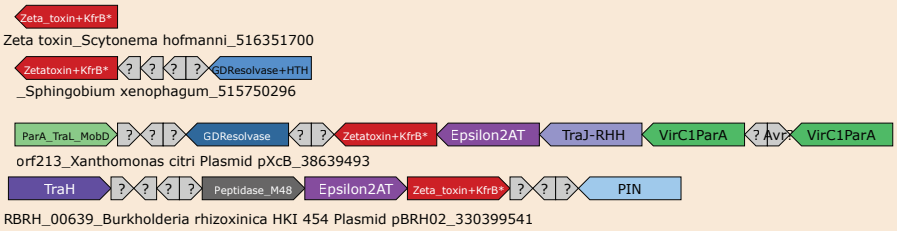


Supplementary Figure 5a extended (arockiasamy)

Relaxase (RCRE) associated



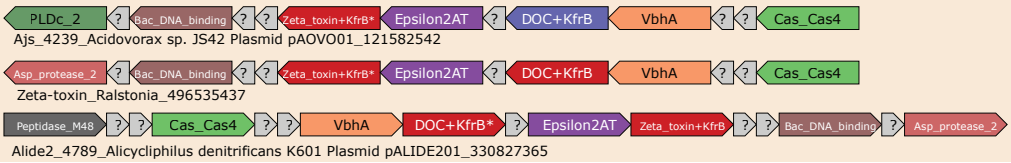
Fused to Zeta toxin



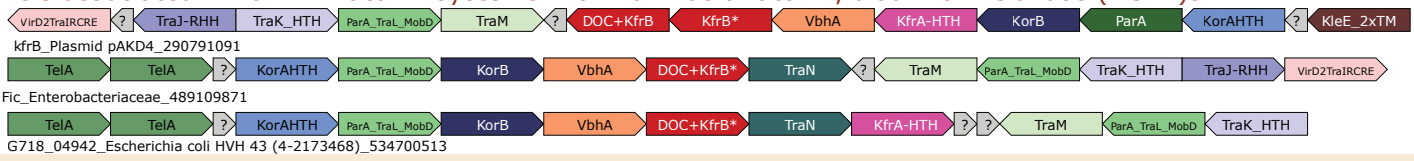
ZetaToxin and Relaxase (RCRE) associated

_Gilvimarinus chinensis_519055168

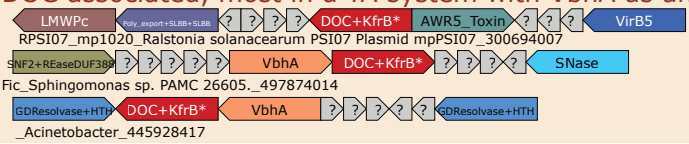
Multiple TA loci



DOC associated in Toxin-Antitoxin systems with VbhA as antitoxin, also with Relaxase (RCRE)s

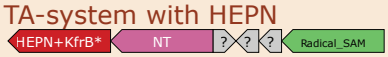


DOC associated, most in a TA system with VbhA as antitoxin

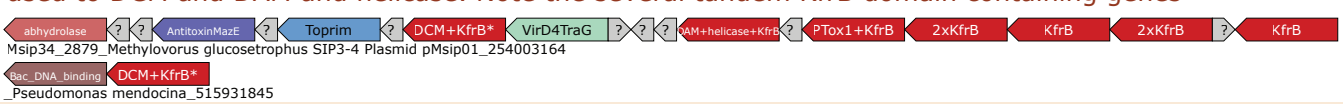


abhydrolase associated, most in a TA system with VbhA

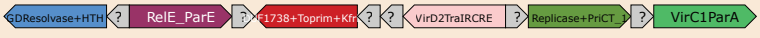
TA-system with HEPN



Fused to DCM and DAM and helicase. Note the several tandem KfrB domain containing genes

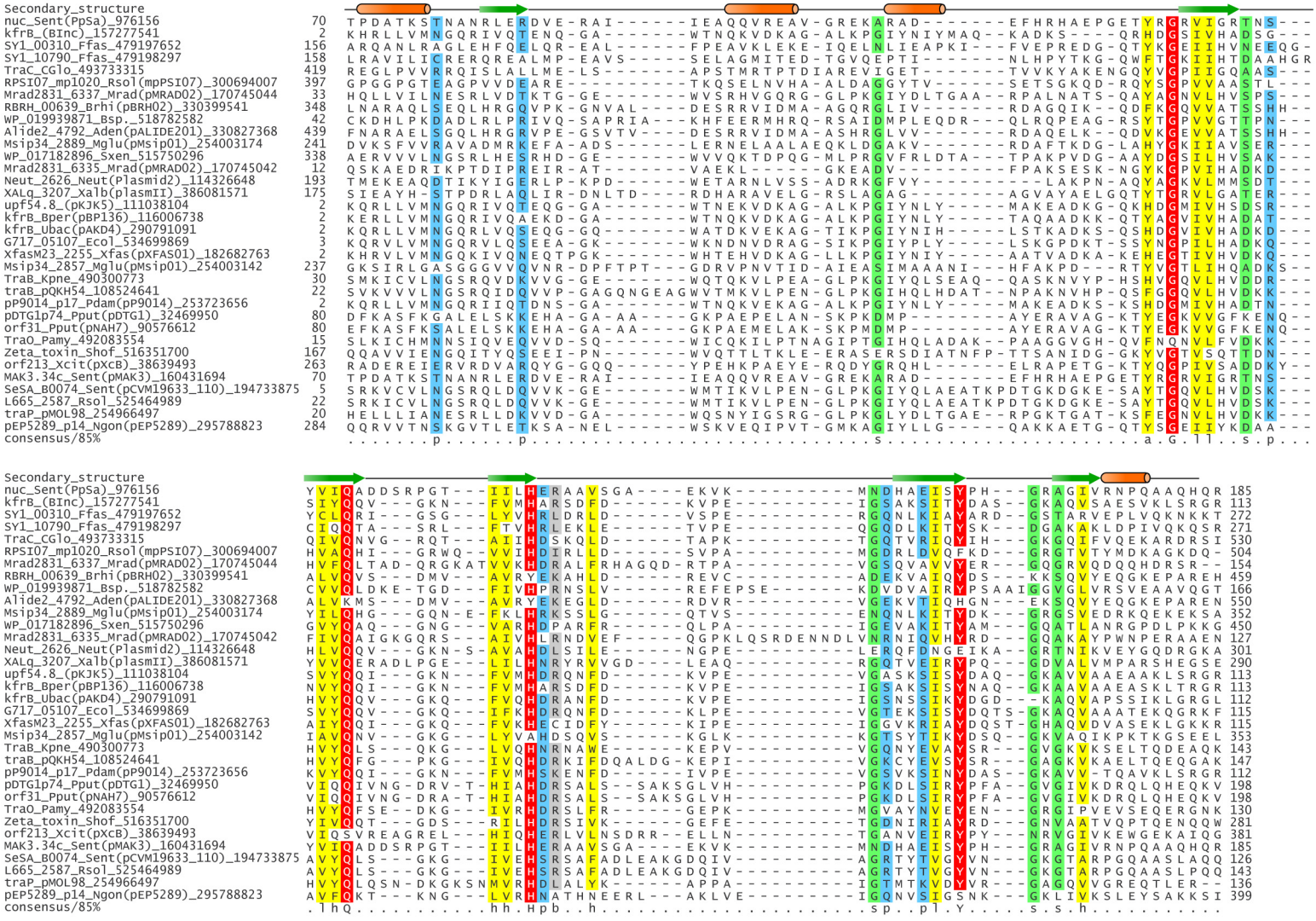


Fused to Toprim and in the neighborhood of a Relaxase (RCRE)

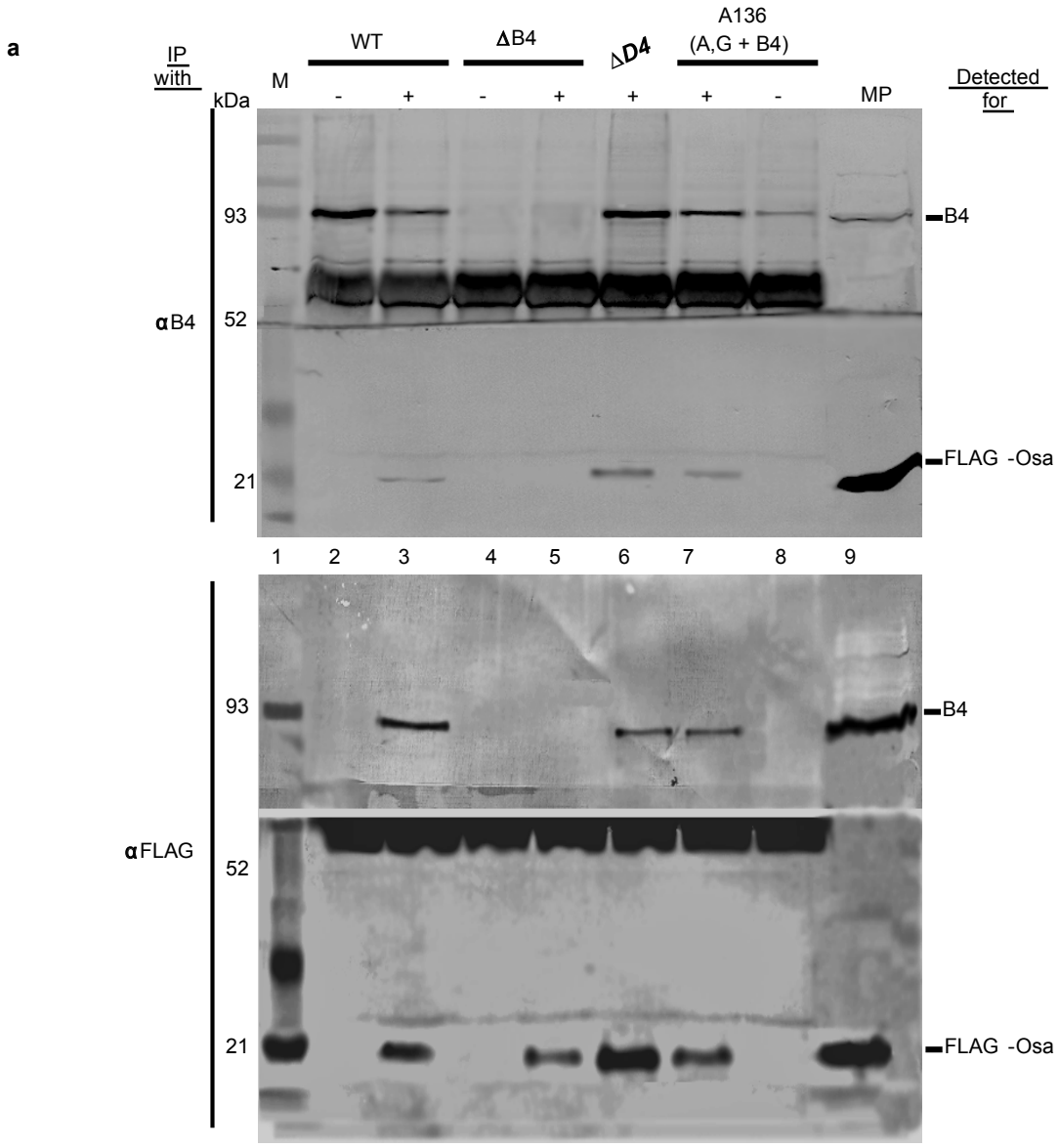


Fused to DUF4314, which is also associated with DNA modification systems

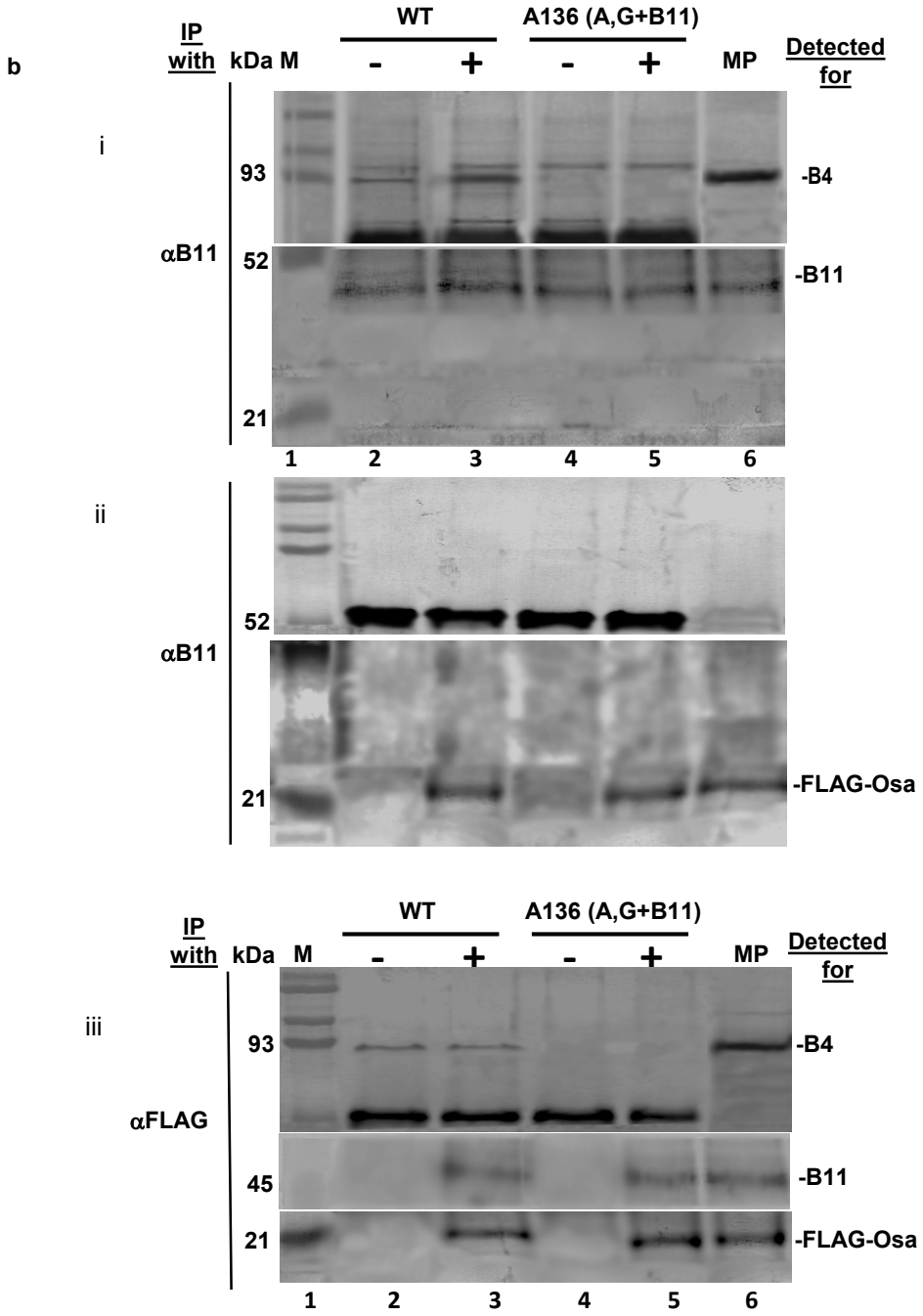
Supplementary Figure 5b (arockiasamy)



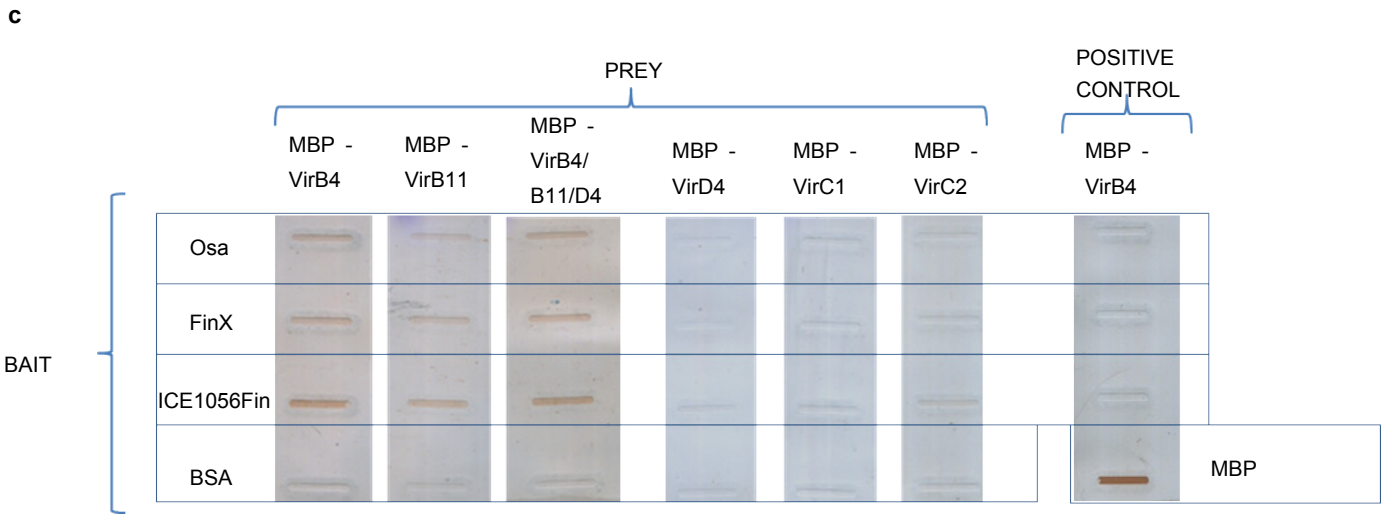
Supplementary figure 5: Operon associations of KfrB and multiple sequence alignment. In the gene neighborhood illustrations (**a**), genes are shown as box-arrows with the arrow head pointing to the 3' gene in the gene neighborhood. KfrB containing genes are colored red. Operons are labeled with gene names, species abbreviations and/or plasmid name and Genbank index numbers (GI) of the KfrB containing genes (marked as asterisk in the gene neighborhood). Gene neighborhoods are grouped based on similarity of associations. In the alignment (**b**), proteins are denoted by their gene names, species abbreviations and Genbank index numbers (GI). Additionally those derived from plasmids are denoted with the plasmid name in brackets. The alignment was colored at 85% consensus. Secondary structure elements are shown above the alignment. The consensus abbreviations and colouring scheme are as in Supplementary figure 1c. Species abbreviations are as follows: Aden : *Alicycliphilus denitrificans*; BInc : *Birmingham IncP-alpha*; Bper : *Bordetella pertussis*; Brhi : *Burkholderia rhizoxinica*; Bsp. : *Bordetella* sp.; CGlo : *Candidatus Glomeribacter*; E : Enterobacteriaceae ; Ecol : *Escherichia coli;* Ffas : *Fretibacterium fastidiosum*; Mglu : *Methylovorus glucosetrophus*; Mrad : *Methylobacterium radiotolerans*; Neut : *Nitrosomonas eutropha*; Ngon : *Neisseria gonorrhoeae*; Pamy : *Pseudomonas amygdali*; Pdam : *Photobacterium damselae*; PpSa : Plasmid pSa; Pput : *Pseudomonas putida*; Rsol : *Ralstonia solanacearum*; Sent : *Salmonella enterica*; Shof : *Scytonema hofmanni*; Spla : Synthetic plasmid; Sxen : *Sphingobium xenophagum*; Ubac : uncultured bacterium; Xalb : *Xanthomonas albilineans*; Xcit : *Xanthomonas citri*; Xfas : *Xylella fastidiosa.*



Supplementary Figure 6a: Uncropped blots of Fig. 4a showing that Osa interacts with VirB11 independent of T4S machine and VirD4. After blocking, membranes i) and ii) were cut into two portions. The top and bottom portions of both i) and ii) were detected for VirB4 and FLAG-Osa respectively.



Supplementary Figure 6b: Uncropped blots of Fig. 4b showing that Osa interacts with VirB11 independent of T4S machine and VirD4. After blocking, membranes were either retained as a single membrane (ii) or cut into two (i) or three (iii) portions. i) The top and bottom membrane portions were detected for VirB4 and VirB11 respectively. ii) The membrane was detected for FLAG-Osa. Since the IgG heavy chain (used in the experiment) got detected at higher intensity even before FLAG-Osa could be detected, the blot was cut into two halves and the top portion was washed. The bottom portion was over exposed to detect for FLAG-Osa. iii) The top portion was used for detection of VirB4, the middle for detection of VirB11 and the bottom piece for detection of FLAG-Osa.



Supplementary Figure 6c : Uncropped blots of Fig. 4c showing Osa interacts with VirB4 and VirB11 *in vitro*.

Supplementary table 1. Plasmids and strains used in this study.

No.	Plasmid	Strain	Description
1	pKA165 (pvirB::osa)	*E. coli* DH5α	*osa* cloned under *virB* promoter, using *Nde*I/*Xho*I
2	pKA197	*E. coli* DH5α	*FLAG-osa* cloned under virB promoter, using *Nde*I/*Xho*I
3	pET28b::osa	*E. coli* DH10β *and* BL21(DE3) plysS	*osa* cloned into pET28b(+) under *Nde*I/*Xho*I with N-terminal His_6
4	pSoup::osa	*A. tumefaciens* LBA4404	*osa* cloned under *virB* promoter using *Hind*III/ *Kpn*I
5	pSoup::osa-K100A	”	*osa-K100A* cloned under *virB* promoter using *Hind*III/*Kpn*I
6	pSoup::osa-R140A	”	*osa-R140A* cloned under *virB* promoter using *Hind*III/*Kpn*I
7	pSoup::osa-KR (100A-R140A)	”	*osa-K100A-R140A* cloned under virB promoter using *Hind*III/*Kpn*I
8	pSoup::osa-penta (D98A-K100A-D136A-T139A-R140A)	”	*osa-D98A-K100A-D136A-T139A-R140A* cloned under virB promoter using *Hind*III/*Kpn*I
9	pSoup::fiwA	”	*fiwA* cloned under virB promoter using *Hind*III/*Kpn*I
10	pSoup::ICE1056Fin	”	*ice1056fin* cloned under virB promoter using *Hind*III/*Kpn*I
11	pSoup::fipA	”	*fipA* cloned under virB promoter using *Hind*III/*Kpn*I sites
12	pSoup::pifC	”	*pifC* cloned under virB promoter using *Hind*III/*Kpn*I sites
13	pET28a::ice1056fin	*E. coli* BL21CodonPlus	Ice1056Fin in pET28a, under *Nco*I/*Xho*I sites with C-His_6 tag
14	pETM41::osa-	*E. coli* Rosetta (DE3) pLysS	MBP tagged Osa in pETM41 under *Nco*I/BamHI
15	pETM41::osa-K100A	*E. coli* Rosetta (DE3) pLysS	MBP tagged Osa-K100A in pETM41 under *Nco*I/*Kpn*I
16	pETM41::osa-R140A	”	MBP tagged Osa-R140A in pETM41 under *Nco*I/*Kpn*I
17	pETM41::osa-KR (K100A-R140A)	”	MBP tagged Osa-K100A-R140A in pETM41 under *Nco*I/*Kpn*I
18	pETM41::osa-penta (D98A-K100A-D136A-T139A-R140A)	”	MBP tagged Osa-K100A-R140A-D136A-T139A-R140A in pETM41 under *Nco*I/*Kpn*I
19	pETM41::virC1	*E. coli* T7 Express I^q	MBP tagged VirC1 in pETM41 under *Nco*I/*Kpn*I
20	pETM41::virC2	”	MBP tagged VirC2 in pETM41 under *Nco*I/*Kpn*I

21	InfR-Co::virD2	*E. coli* Rosetta (DE3) pLysS	VirD2 in InfR-Co in the second cassette with C-His_6 tag
22	pETM41::Δ1-86virD4	”	MBP tagged VirD4 (87-543) in pETM41under *Nco*I/*Kpn*I
23	pETM41::Δ1-424virB4	*E. coli* T7 Express I^q	MBP tagged VirB4 (425-743) in pETM41under *Nco*I/*Kpn*I
24	pETM41::virB11	*E. coli* Rosetta (DE3) pLysS	MBP tagged VirB11in pETM41 under *Nco*I/*Kpn*I
25	InfR-Co::P1 parB	*E. coli* T7 Express I^q	His_6-tagged P1 ParB in InfR-Co first cassette
26	InfR-Co::RK2 korB	*E. coli* Rosetta (DE3) pLysS	His_6-tagged RK2 KorB in InfR-Co first cassette

pETM41 was obtained from EMBL protein expression and purification core facility. InfR-Nhis, InfR-Chis and InfR-Co are in-house modified vectors from pRSF-Duet1 (Novagen) cotaining In-Fusion (Clonetech) cloning cassettes. InfR-Nhis and InfR-Chis both have an In-Fusion cloning cassette with TEV protease cleavable His_6-tag at the N and C-terminus, respectively. InfR-Co has two In-Fusion cassettes with both TEV protease cleavable N-terminal His_6-tag in the first cassette and C-terminal His_6-tag in the second cassette.

Supplementary table 2. List of oligos used in this study.

Primer	Seequence	Purpose
osa-R140A-FP	GGCATGACCGCTTCAATGTGG	To generate *osa-R140A*
osa-R140A-RP	ATCAGTGAAACACACCGCGAAATG	"
osa-K100A-FP	AAAGACCGGGCATATCCGTCG	To generate *osa-K100A* mutant and *osa-K100A-R140A* mutant
osa-K100A-RP	AGGCATGTCCTGCTTCAACGG	"
osa-D98A-InK100A-FP	ATGCCTAAAGCCCGGGCATAT	To generate *osa-D98A-K100A-R140A* from *Osa-K100A-R140A*
osa-D98A-InK100A-RP	GTCCTGCTTCAACGGGCAGGC	"
osa-D136A-T139A-InR140A-FP	TTCACTGCTGGCATGGCCGCTTCA	To generate *osa-D98A-K100A-D136A-T139A-R140A* from *osa-D98A-K100A-R140A*
osa-D136A-T139A-InR140A-RP	ACACACCGCGAAATGGCCCAGAAT	"
fiwA-FP	AGACCATGGCTATGCTGACACGGTTGAAG	PCR amplification of *fiwA* from pRP4* and cloning into pAK165
fiwA-RP	AGACTCGAGTTATCAGAGTCCGCGGCCTTT	"
fipA-FP	AGACCATGGCTATGATGGAGCAGATCGAC	PCR amplification of *fipA*from pKM101+ and cloning into pAK165
fipA-RP	AGACTCGAGTTATCACAGTGTAAGCAGGCG	"
ice1056fin-FP	AGACCATGGCTATGATAAAACTGACCCCT	PCR amplification of *ice1056fin* from ICE*Hin*1056[#] and cloning into pAK165
ice1056fin-RP	AGACTCGAGTTATTACAAGGGGTATTTTTCTCC	"
pifC-FP	AGACCATGGCTATGATGCTAAGCCAGCTT	PCR amplification of *pifC* of plasmid F' from *E. coli* Rosetta-gami (DE3) plysS (Novagen) and cloning into pAK165
pifC-RP	AGAGTCGACTTATTACAGATCTCCGTACAGGC	"
ice1056fin -pET28a-FP	AGACCATGGCTATGATAAAACTGACCCCT	PCR amplification of *ice1056fin* from ICE*Hin*1056 and cloning into pET28a
ice1056fin -pET28a-RP	AGACTCGAGCAAGGGGTATTTTTCTCC	"
VirD4 (87-543) NhisFP	TACTTCCAGTCCATGGGACTGCGCAATCAGAAG	*virD4* cloning into InfR-Co for overexpression and purification
VirD4 (87-543)	AGAGTTATCAACATATGTTATTA	"

NhisRP	CTGGCGTTCAAAAAGC	
VirB4 (425-743) NhisFP	TACTTCCAGTCCATGGATGTTGG CATGACGG	*virB4* cloning into InfR-Co for for overexpression and purification
VirB4 (425-743) NhisRP	AGAGTTATCAACATATGTTATTA CCGCAGATCAAATTC	"
VirB11NhisNcoIFP	TACTTCCAGTCCATGGAAGTGG ATCCGCAACTAC	*virB11* cloning into InfR-Co for for overexpression and purification
VirB11NhisNdeIRP	AGAGTTATCAACATATGTTATTG ATTTAGGAGATCGCC	"
Virc1NhisNco1FP	TACTTCCAGTCCATGGCTCAACT TTTGACGTTTTGCTC	*virC1* cloning into InfR-Nhis for for overexpression and purification
Virc1NhisNde1RP	AGAGTTATCAACATATGTTATTA AGCCTCCAAGATTT	"
Virc2 NhisNco1FP	TACTTCCAGTCCATGGCAATTCG CAAGCCCGCGTT	*virC2* cloning into InfR-Nhis for for overexpression and purification
Virc2 NhisNde1RP	AGAGTTATCAACATATGTTATTA CCAATTCCTCGATG	"
VirD2 NhisNco1FP	TACTTCCAGTCCATGGCTCCCGA TCGCGCTCAAGTA	*virD2* cloning into InfR-Co for for overexpression and purification
VirD2 NhisNde1RP	AGAGTTATCAACATATGTTATTA GGTCCCCCCGCGCC	"
Osa NcoI FP	TTTCAGGGCGCCATGGCTTTGCT ACGGCGGCGGTGTCG	*Osa, osa-K100A, osa-R140A, osa-RK* and *osa-penta* cloning into pETM41 for overexpression and purification
Osa BamHI RP	GCTCGAATTCGGATCCCTAGAT CTTCCTGCATTGCTCA	*"*
P1ParB NcoInhisFP	TACTTCCAGTCCATGGCTTCAAA GAAAAAC	*p1parB* cloning into InfR-Co for for overexpression and purification
P1ParB KpnINhisRP	GTTAATTAACTCGAGGGTACCT CAAGGCTTCGGCT	"
KorB NcoINhisFP	TACTTCCAGTCCATGGCTACTGC GGCTCAAG	*rk2korB* cloning into InfR-Co for for overexpression and purification
KorB KpnINhisRP	GTTAATTAACTCGAGGGTACCT CAGCCCTCGATGAGC	"
FLAG-Osa-FP	GCGCTAACGATGCATATGGATT ACAAGGATGACGATGACAAGTT GCTACGGCGG	For *in vivo* protein-protein interaction studies
FLAG-Osa-RP	AAAGCGCAGGGACTCGAGTCAC TATTCTAGAATCTTCCTGCATTG	"
90-mer oligo with RB and LB	TTGGCAGGATATATTGTGGTGT AAACATTGTCGTTTCCCGCCTTC AGTTTAAACTATCAGTGTTTGAC AGGATATATTGGCGGGTAAACC TAAGAGAAAAGAGCGTTTA	For ssDNA nuclease assays, and for T-DNA-VirD2 complex preparation

[*]DSM collection No. 3876, [+]DSM collection No. 9496, [#]Integrative Conjugative Element ICE*Hin*1056 isolated from *Haemophilus influenzae*. DSM - Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (www.dsmz.de).

Supplementary table 3. Composition of buffers used for protein purification.

Name of the buffer	Buffer composition	pH	NaCl (mM)	Imidazole (mM)	βME (mM)	DTT (mM)	Glycerol (%)	PMSF (mM)
Lysis buffer 1*	50 mM Tris-HCl	8.0	500		3			1
Lysis buffer 2#	25 mM Tris-HCl	"	150		2		10	1
A	"	″	500	10	2			
B	"	″	"	500	2			
C	"	″	200		5			
D	"	″	750			2		
E	"	″	500		5			
F@	"	″	1000		5			
G	"	″	500			2		
H$	20 mM Tris-HCl	7.4	100					
I	25 mM Tris-HCl	8.0	10			2		
J	"	″	1000			2		
K	50 mM Tris-HCl	″	300	10			10	1
L	50 mM Tris-HCl	″	"	1000			10	1
M	50 mM Na-phosphate	″	50				10	
N	50 mM Na-phosphate	″	1000				10	

*-contains 1 mM Benzamidine-HCl, #-contains 1 mM Benzamidine-HCl, 10 µg/ml DNase I and 100 µg/ml Hen Egg White Lysozyme, @-contains 20 mM D-maltose, $-contains 1 mM EDTA.