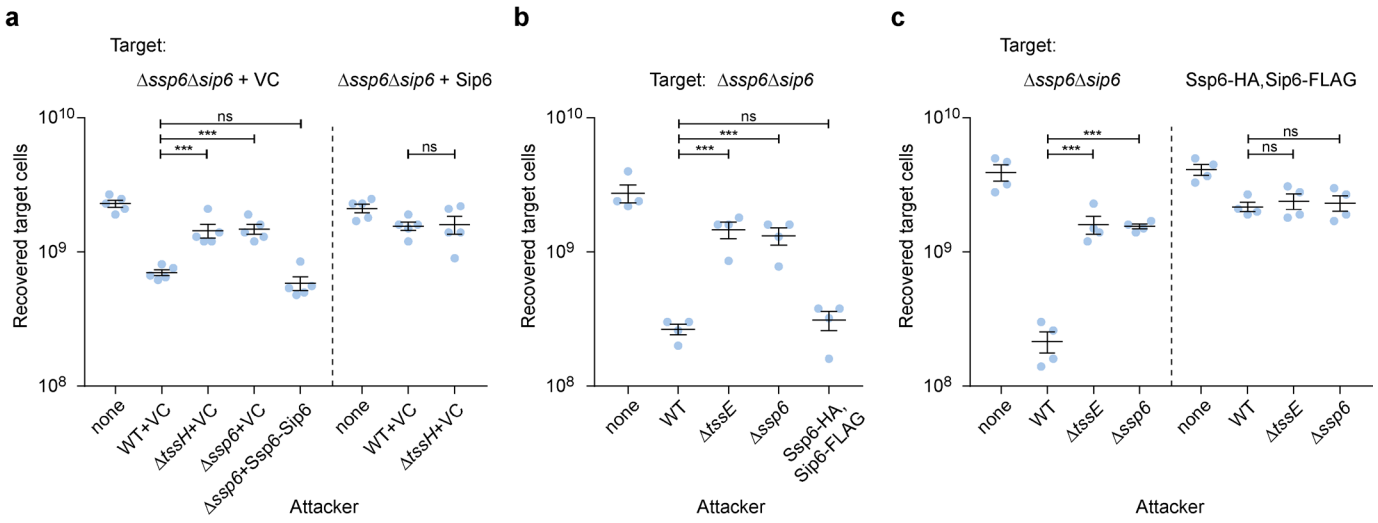
Supplementary Information

A family of Type VI secretion system effector proteins that form ion-selective pores

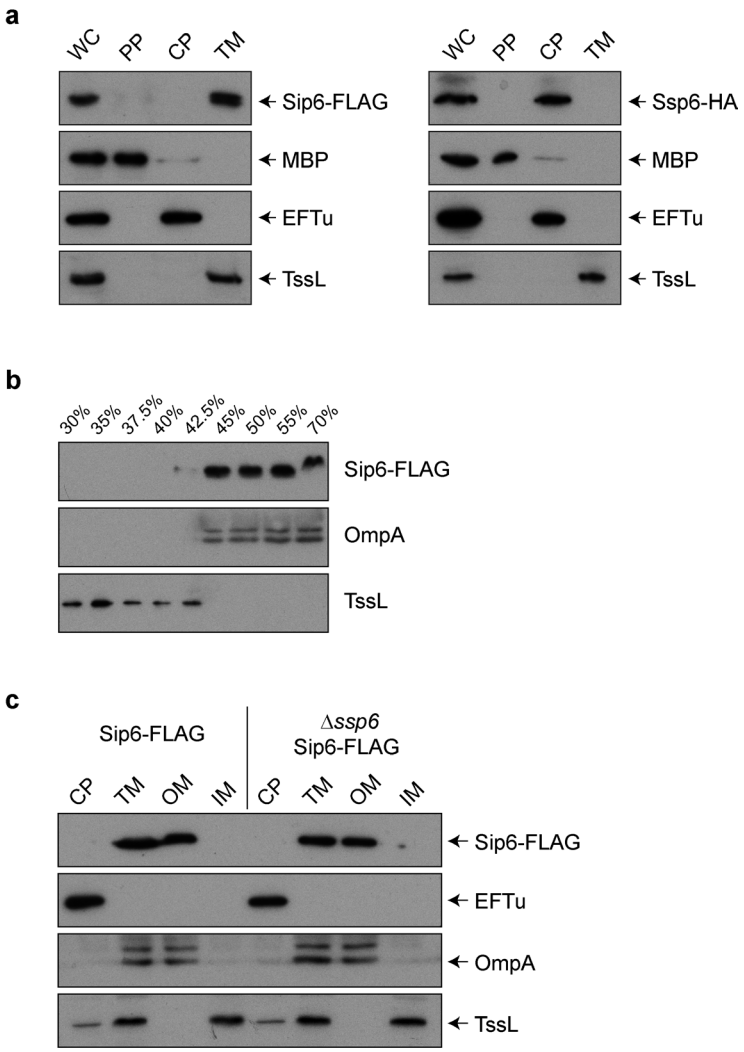
Mariano *et al*.

Supplementary Figure 1



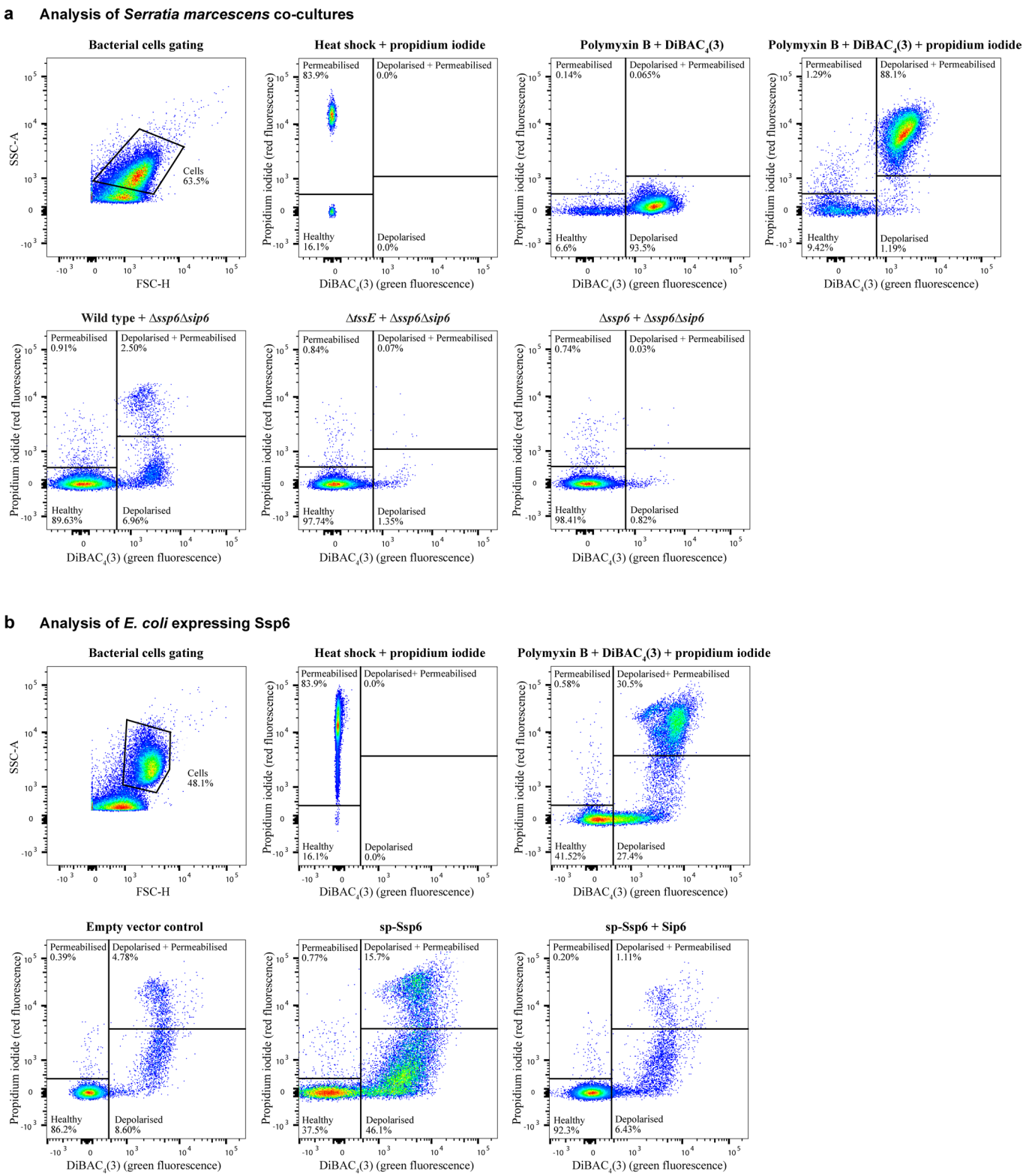
Supplementary Figure 1. Complementation and functionality of strains carrying Ssp6 and Sip6 deletions or epitope tag fusions. (a) Recovery of target strain *S. marcescens* Db10 Δ*ssp6*Δ*sip6* carrying the empty vector control (+VC, pSUPROM) or a plasmid directing the expression of Sip6-HA (+Sip6), following 7.5 h co-culture with wild type (WT) or mutant strains (Δ*tssH*, Δ*ssp6*) of Db10 carrying the empty vector control (+VC, pBAD18-Kan) or a plasmid directing the expression of Ssp6 and Sip6 (+Ssp6-Sip6). Expression of Ssp6 and Sip6 was induced by addition of 0.02% L-arabinose; Sip6 was included in the Ssp6 construct to prevent toxicity from overexpression of Ssp6. (b) Recovery of the Δ*ssp6*Δ*sip6* mutant following co-culture with wild type *S. marcescens* Db10, the Δ*tssE* mutant and the strain encoding Ssp6-HA and Sip6-FLAG fusion proteins at the normal chromosomal location. (c) Recovery of the wild type and the strain encoding Ssp6-HA and Sip6-FLAG fusion proteins as targets, when co-cultured with wild type, Δ*tssE* or Δ*ssp6* attackers. All target strains used in the study represent streptomycin-resistant derivatives of the strain indicated. In all parts, individual data points are overlaid with the mean +/- SEM (n=4 biological replicates, or n=5 in part a; *** indicates $p<0.001$, ns indicates not significant, one-way ANOVA with Dunnett's test; except for the Δ*ssp6*Δ*sip6* + VC target in part a, where a two-tailed t-test was used); none, target cells incubated with sterile media alone. Source data are provided as a Source Data file.

Supplementary Figure 2



Supplementary Figure 2. Subcellular localisation of Sip6 and Ssp6. (a) Cells of *S. marcescens* Db10 carrying chromosomally-encoded Sip6-FLAG (left hand panel) or Ssp6-HA (right hand panel) were fractionated into whole cell (WC), periplasm (PP), cytoplasm (CP) and total membrane (TM) fractions and the fractions were analysed by immunoblot detection of the FLAG epitope, the HA epitope, MBP (periplasmic control protein), EFTu (cytoplasmic control protein) and TssL (integral membrane protein). (b) Total membrane fractions from *S. marcescens* Db10 carrying Sip6-FLAG were separated by discontinuous sucrose gradient centrifugation and analysed by immunoblot detection of OmpA (outer membrane control protein) and TssL (inner membrane control protein). The percentage of sucrose in each fraction is given along the top. (c) Cells of *S. marcescens* Db10 carrying Sip6-FLAG in the wild type or the Δ*ssp6* background were fractionated into cytoplasm, total membrane, inner membrane (IM) and outer membrane (OM) fractions and the fractions analysed by immunoblot detection using antibodies as above. In all parts, the cells were grown in liquid culture, preventing productive T6SS-mediated translocation of effectors between cells. Therefore the cells can be considered as 'attackers' and the localisations as 'pre-secretion', since any secreted effectors will be released to the media. Source data are provided as a Source Data file.

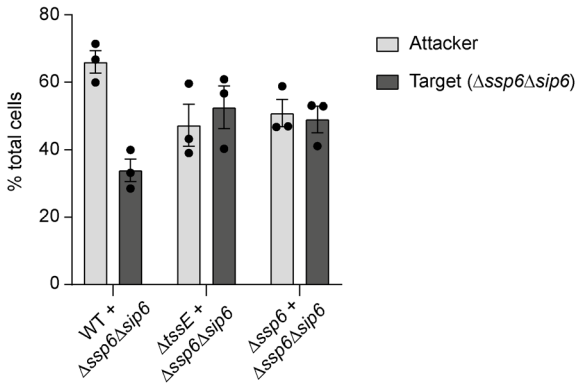
Supplementary Figure 3



Supplementary Figure 3. Examples of individual flow cytometry experiments from the analyses presented in Figure 3. (a) Analysis of *S. marcescens* co-culture experiments, as Figure 3a. Top panels illustrate the gating strategy. Bacterial cells were selected using side scatter (SSC-A) vs. forward scatter (FSC-H). Cells subjected to heat shock treatment and stained with propidium iodide only were used to

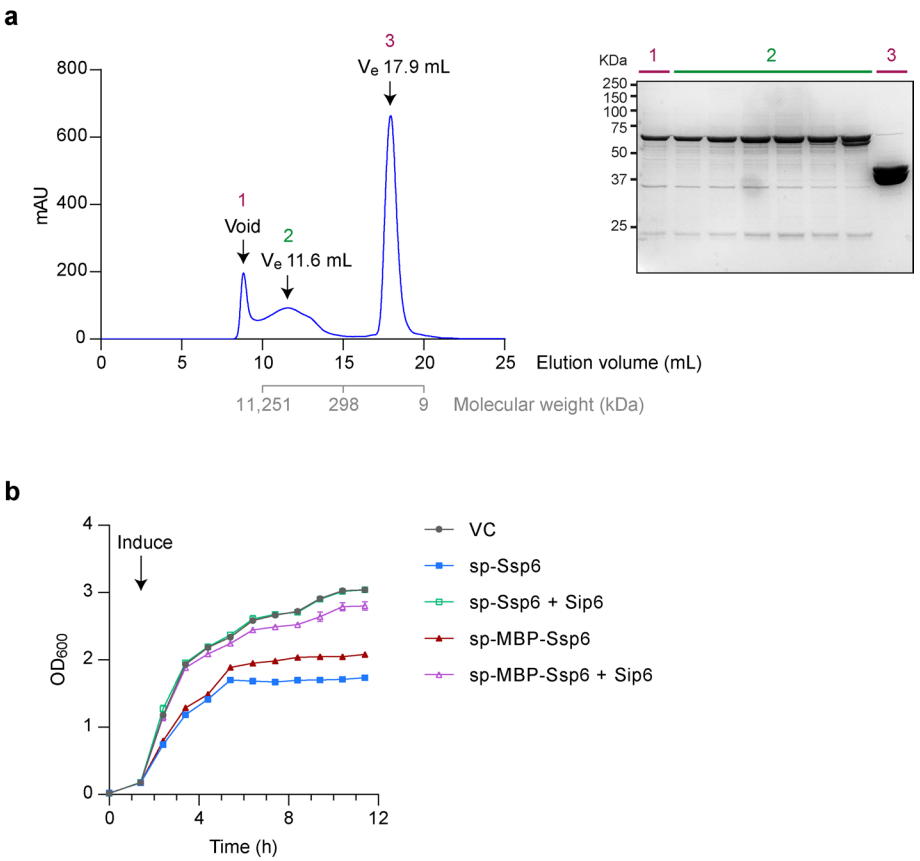
define the permeabilized cells quadrant (red fluorescence), whilst treatment with 300 μg/mL polymyxin B and staining with $DiBAC_4(3)$ only was used to define the depolarised cells quadrant (green fluorescence). Cells treated with polymyxin B and stained with $DiBAC_4(3)$ and propidium iodide were used to establish the quadrant for cells that are simultaneously depolarised and permeabilized (green and red fluorescence). Bottom panels show representative plots from experiments involving co-culture of the Ssp6-susceptible target strain Δ*ssp6*Δ*sip6* with wild type (WT) or control (Δ*ssp6* or Δ*tssE*) attacker strains of *S. marcescens* Db10, followed by simultaneous staining with propidium iodide and $DiBAC_4(3)$. (b) Analysis of *E. coli* cells expressing sp-Ssp6, as Figure 3b. Top panels illustrate the gating strategy. Bacterial cells were selected using SSC-A vs. FSC-H. Cells subjected to heat shock treatment and stained with propidium iodide only were used to define the permeabilized cells quadrant (red fluorescence), whilst treatment with 2 μg/mL polymyxin B and staining with both propidium iodide and $DiBAC_4(3)$ defined the quadrant of cells that are simultaneously depolarised and permeabilized (green and red fluorescence) and determined the separation from the quadrant containing cells that are depolarised but not permeabilised (green fluorescence). Bottom panels show representative plots from experiments analysing *E. coli* cells expressing sp-Ssp6 or sp-Ssp6 + Sip6, or healthy control cells carrying the empty vector (VC).

Supplementary Figure 4



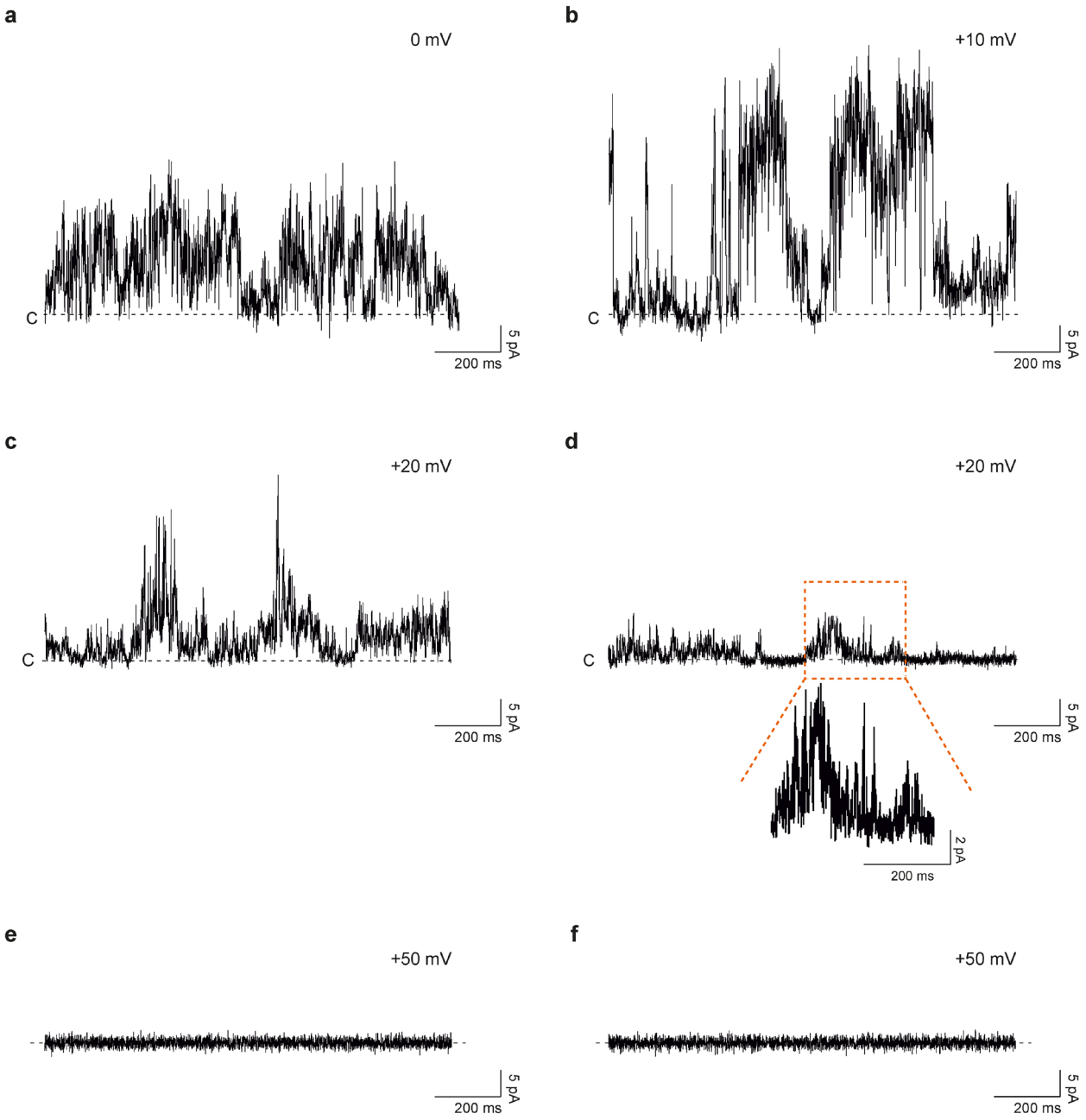
Supplementary Figure 4. Quantification of the relative proportion of attacker and target cells following co-culture under the conditions used for membrane potential and permeability analysis in Figure 3a. The Ssp6-susceptible target strain, Δ*ssp6*Δ*sip6* expressing cytoplasmic GFP, was co-cultured with wild type (WT) or mutant (Δ*ssp6* or Δ*tssE*) attacker strains expressing cytoplasmic mCherry for 4 h at an initial ratio of 1:1. Cells were recovered, imaged by fluorescence microscopy and numbers of green (GFP) and red (mCherry) cells counted using OMERO mTools. The relative numbers of attacker and target cells for each condition following co-culture is represented as a percentage of the total cell population. Around 1500 cells in total were counted for each condition in each replicate. Bars show mean +/- SEM, with individual data points superimposed (n=3 independent experiments). Source data are provided as a Source Data file.

Supplementary Figure 5



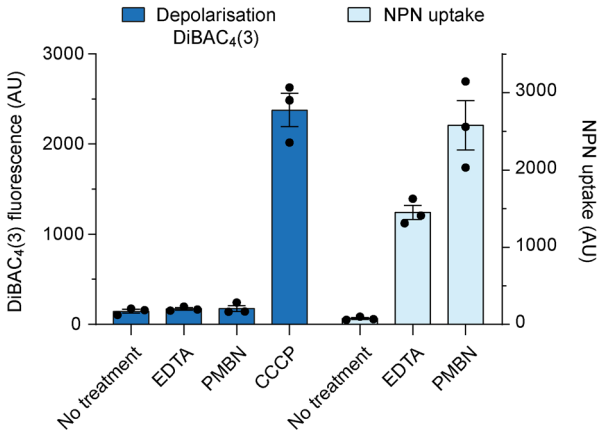
Supplementary Figure 5. MBP-Ssp6 is oligomeric and retains toxin function. (a) Size exclusion chromatography of MBP-Ssp6, following its initial isolation by dextrin affinity purification, using a calibrated Superose 6 Increase 10/300 GL column (GE Healthcare). For each peak, the elution volume (V_e) is indicated. Analysis of fractions eluted during size exclusion chromatography by SDS-PAGE and Coomassie staining is shown on the right, with peak 2 (green fractions) containing the MBP-Ssp6 used for subsequent analyses. Peak 3 contains MBP only. (b) Growth of *E. coli* MG1655 carrying empty vector (VC, pBAD18-Kn) or plasmids directing the expression of the MBP-Ssp6 fusion protein with an N-terminal OmpA signal peptide (sp-MBP-Ssp6) or native Ssp6 with signal peptide (sp-Ssp6), each with or without co-expression of Sip6 (+ Sip6), in LB containing 0.2% L-arabinose. Point show mean ± SEM (n=3 biological replicates). Source data are provided as a Source Data file.

Supplementary Figure 6



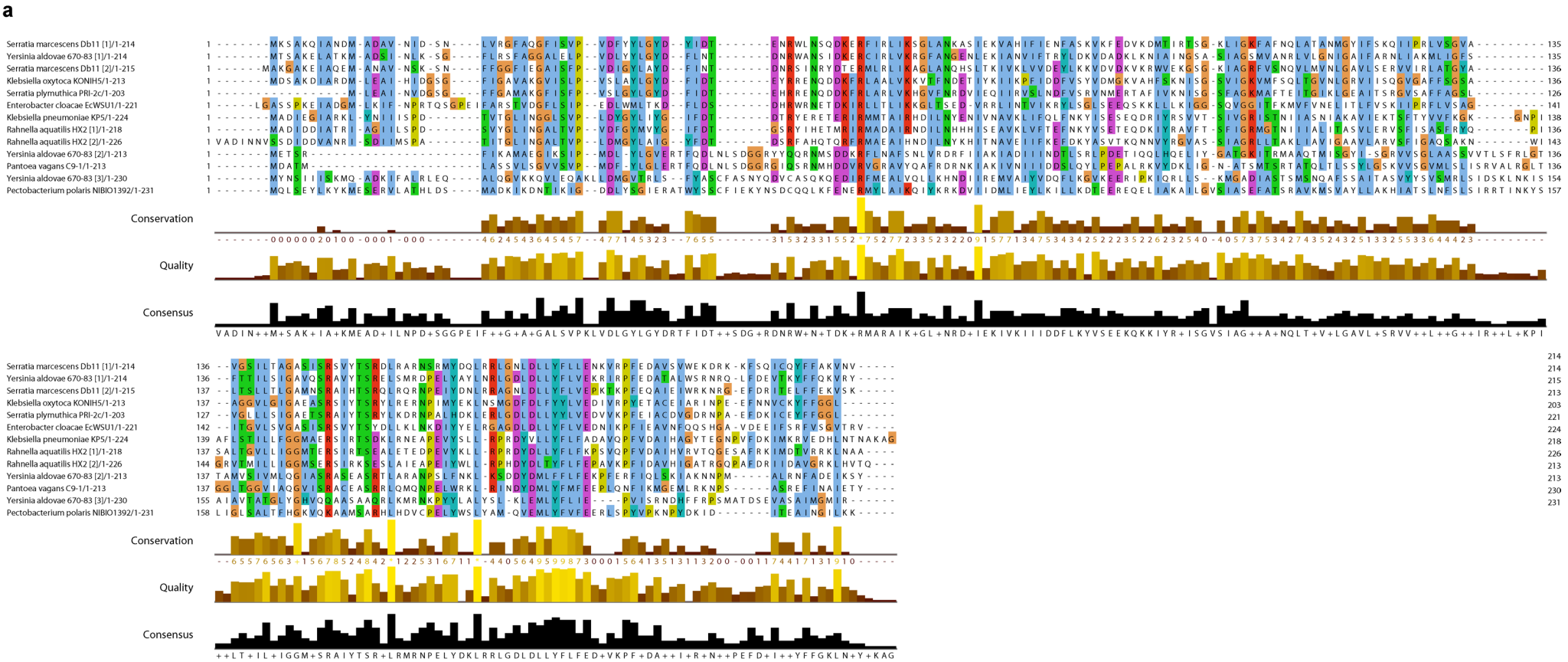
Supplementary Figure 6. Gating characteristics of Ssp6. (a,b) Representative gating of MBP-Ssp6 in a KCl gradient at a holding potential of 0 mV (panel a) and +10 mV (panel b). MBP-Ssp6 gates to more than one open state. When multiple Ssp6-MBP proteins incorporate into the bilayer coordinated gating is observed. (c, d) Examples of MBP-Ssp6 current fluctuations measured in bi-ionic conditions where 210 mM NaCl (panel c) or $CaCl_2$ (panel d) were added to the *cis* chamber, with 210 mM KCl in the *trans* chamber. In these conditions, MBP-Ssp6 channels retained coordinated and unstable gating. In panel d, a magnification of the MBP-Ssp6 gating is shown to aid clarity. In panels a-d, the traces shown are representative of four (panels a, b) or three (panels c, d) independent experiments, and the dotted line indicates the closed (C) state of the pore. (e, f) Measurement of current fluctuations following addition of purification buffer (panel e) or MBP alone (panel f); the dotted line shows the baseline zero current. No current fluctuations were observed in three independent experiments.

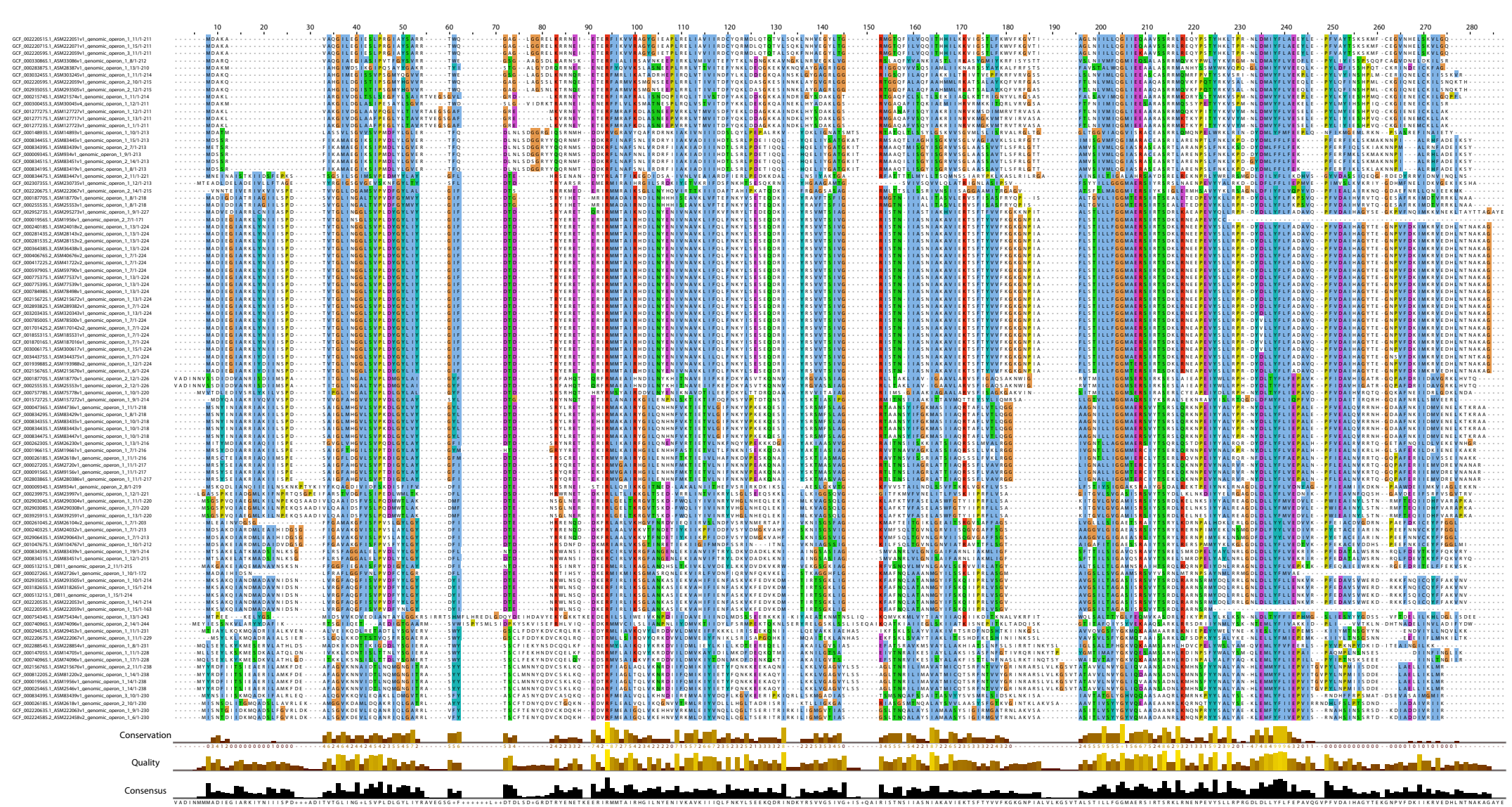
Supplementary Figure 7



Supplementary Figure 7. Treatments disrupting the integrity of the outer membrane do not lead to depolarisation of the inner membrane. Cells of *E. coli* MG1655 were treated with EDTA or polymyxin B nonapeptide (PMBN) to disrupt the outer membrane, or CCCP as a control to dissipate the inner membrane potential, and membrane depolarisation was measured as $DiBAC_4(3)$ fluorescence. To confirm the activity of EDTA and PMBN under these conditions, outer membrane permeabilisation (NPN uptake) was measured in parallel, in both cases using a 96-well plate fluorimeter based assay. Healthy cells with no treatment represent the negative control. Bars show mean +/- SEM, with individual data points superimposed (n=3 independent experiments). Source data are provided as a Source Data file.

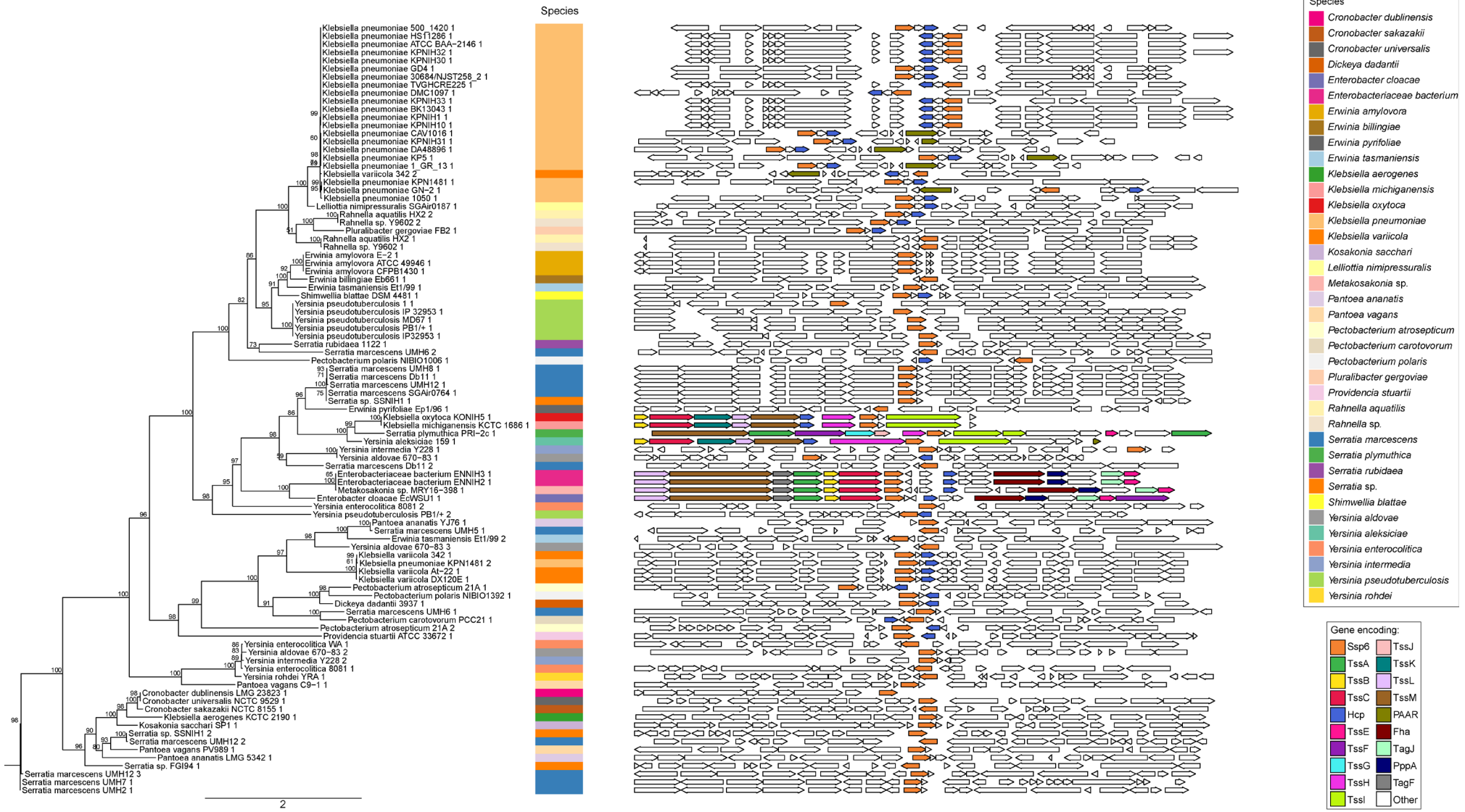
Supplementary Figure 8




b
Conservation
Quality
Consensus

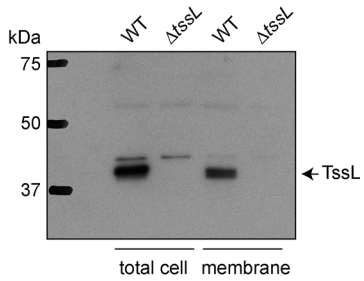
Supplementary Figure 8. Multiple sequence alignment of Ssp6 homologues. The amino acid sequence alignments of the Ssp6-like proteins used to generate the trees depicted in Figure 6 (panel a, selected examples) and Supplementary Figure 9 (panel b, all homologues identified) are visualised using Jalview with the ClustalX colour scheme (http://www.jalview.org). Details of the entries in (b), including the organism encoding the homologue, can be found in Supplementary Data 1.

Supplementary Figure 9



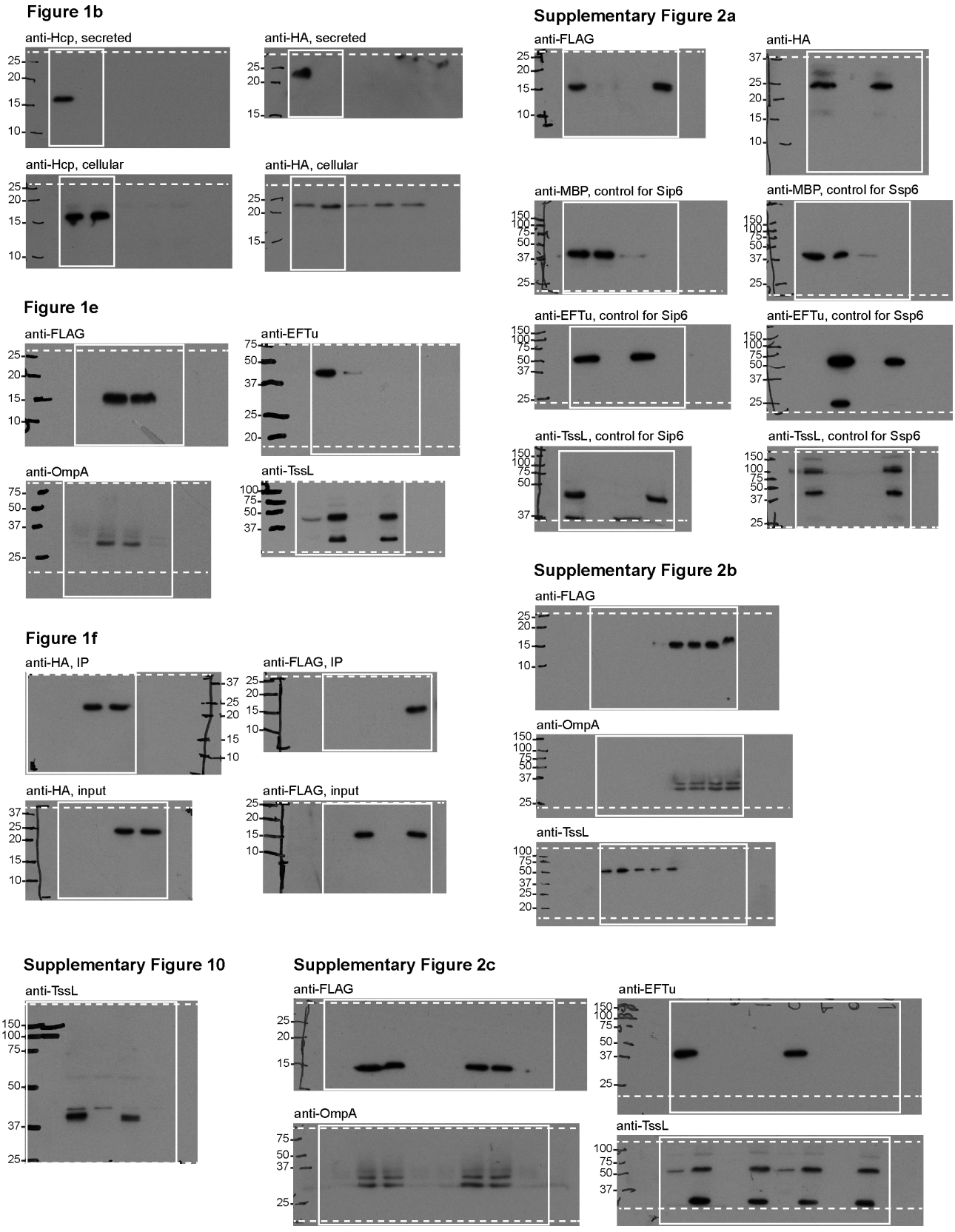
Supplementary Figure 9. Full set of Ssp6-like proteins identified through bioinformatics analysis. Phylogenetic tree and genetic context of homologues of Ssp6 identified using a HMMER search of complete, published bacterial genome sequences. Representative examples taken from this set are depicted in Figure 6 and details of the genome sequences and identified homologues are given in Supplementary Data 1. Note that small open reading frames downstream of *ssp6* encoding putative immunity proteins are frequently missed by the automated annotation; hence absence of such an open reading frame in the schematic does not imply such an immunity does not exist. Manual examination of a selection of such cases revealed that a candidate open reading frame downstream of the *ssp6*-like gene was present in each case as predicted. Genes whose boundaries extend beyond the selected region of interest are also not depicted.

Supplementary Figure 10



Supplementary Figure 10. Validation of custom anti-TssL antibody. Total cellular protein and total membrane protein samples were prepared from the wild type (WT) and a Δ*tssL* mutant of *S. marcescens* Db10, separated by SDS-PAGE and subjected to immunoblotting with a rabbit polyclonal antibody raised against the purified periplasmic domain of TssL (SMDB11_2254). Source data are provided as a Source Data file.

Supplementary Figure 11



Supplementary Figure 11. Uncropped immunoblot images. White boxes indicate lanes included in Figures, dashed lines indicate where membranes were cut, and numbers correspond to molecular weight markers (kDa).

Supplementary Table 1. Strains and Plasmids used in this study.

Name	Description/ genotype	Source / Reference
Strains		
Serratia marcescens		
Db10	Wild type	[1]
SJC11	Db10 Δ*tssE* (*SMDB11_2271*)	[2]
SJC3	Db10 Δ*tssH* (*SMDB11_2274*)	[2]
KT74	Db10 Δ*ssp6* (*SMBD11_4673)*	This study
KT75	Db10 Δ*ssp6*Δ*sip6* (*SMBD11_4673, SMBD11_4672A)*	This study
KT77	Db10 Δ*ssp6*Δ*sip6*, Sm-resistant derivative	This study
KT121	Db10 Ssp6-HA (encodes Ssp6 [*SMDB11_4673*] with a C-terminal HA tag at the native chromosomal location)	This study
KT123	Db10 Ssp6-HA, Δ*tssE*	This study
KT101	Db10 Sip6-FLAG (encodes Sip6 [*SMDB11_4672A*] with a C-terminal 3xFLAG tag at the native chromosomal location)	This study
GM021	Db10 Ssp6-HA, Sip6-FLAG	This study
AO07	Db10 Δ*lacZ*::P_{T5}-*mCherry-kn*^{R} (*lacZ*, *SMDB11_2462*). Encodes cytoplasmic mCherry, IPTG-inducible and expressed constitutively at a low level.	This study
AO08	Db10 Δ*tssE*, Δ*lacZ*::P_{T5}-*mCherry-kn*^{R}	This study
GM24	Db10 Δ*ssp6*, Δ*lacZ*::PT5-*mCherry-kn*^{R}	This study
SAN195	Db10 Δ*lacZ*::P_{T5}-*gfpmut2-kn*^{R}. Encodes cytoplasmic GFP, IPTG-inducible and expressed constitutively at a low level.	[3]
GM04	Db10 Δ*ssp6*Δ*sip6*, Δ*lacZ*::P_{T5}-*gfpmut2-kn*^{R}.	This study
GM143	Db10 Δ*0810* (*SMDB11_0810)*	This study
GM141	Db10 Δ*0810*Δ*0809* (*SMDB11_0810, SMDB11_0809*)	This study
GM144	Db10 Δ*ssp6*, Δ*0810*	This study
GM142	Db10 Δ*ssp6*Δ*sip6*, Δ*0810*Δ*0809*	This study
GM145	Db10 Δ*0810*Δ*0809*, Sm-resistant derivative	This study
GM146	Db10 Δ*ssp6*Δ*sip6,* Δ*0810*Δ*0809*, Sm-resistant derivative	This study
GM160	Db10 Δ*ssp6*, Sip6-FLAG	This study
Escherichia coli		
MG1655	Wild type (model K-12 strain)	[4]
C43(DE3)	Protein overexpression strain; chromosomal λDE3 encodes IPTG-inducible T7 RNA polymerase F$^-$ *ompT hsdS* ($r^{B-}m^{B-}$) *gal dcm* λ(DE3)	Lucigen
CC118λ*pir*	Cloning host and donor strain for pKNG101-derived allelic exchange plasmids (λ*pir*)	[5]
HH26 pNJ5000	Mobilizing strain for conjugal transfer	[6]
Plasmids		
pSUPROM	Vector for constitutive expression of cloned genes under the control of the *E. coli tat* promoter (Kn^R)	[7]
pBAD18-Kn	Arabinose-inducible expression vector; gene of interest is cloned downstream of the P_{ara} promoter (Kn^R)	[8]
pKNG101	Suicide vector for allelic exchange (Sm^R, *sacBR, mobRK2, oriR6K*)	[9]
pNIFTY-MBP	Expression vector for T7 polymerase-dependent expression of recombinant proteins fused with N-terminal MBP-His_6 (Ap^R)	[10]

pSC1273	Coding sequence for Sip6-HA (SMDB11_4672A) in pSUPROM	This study
pSC1235	Coding sequence for Ssp6 (SMDB11_4673) in pBAD18-Kn	[11]
pSC1236	Coding sequence for $OmpA_{SP}$-Ssp6 fusion protein in pBAD18-Kn	[11]
pSC1256	Coding sequences for Ssp6 + Sip6 in pBAD18-Kn	This study
pSC1271	Coding sequence for $OmpA_{SP}$-Ssp6 + Sip6 in pBAD18-Kn	This study
pSC1587	Coding sequence for $OmpA_{SP}$-MBP-Ssp6 + Sip6 in pBAD18-Kn	This study
pSC1597	Coding sequence for $OmpA_{SP}$-MBP-Ssp6 in pBAD18-Kn	This study
pSC2549	Coding sequence for $OmpA_{SP}$-SMBD11_0810 in pBAD18-Kn	This study
pSC2561	Coding sequence for $OmpA_{SP}$-SMBD11_0810 + SMDB11_0809 in pBAD18-Kn	This study
pSC1270	Coding sequence for Ssp6 in pNIFTY-MBP	This study
pSC1264	pKNG101-derived allelic exchange plasmid for the generation of chromosomal in-frame Δ*ssp6* deletion	This study
pSC1266	pKNG101-derived allelic exchange plasmid for the generation of chromosomal in-frame Δ*ssp6*Δ*sip6* deletion	This study
pSC1311	pKNG101-derived allelic exchange plasmid for the generation of Ssp6-HA allele at the normal chromosomal location	This study
pSC1290	pKNG101-derived allelic exchange plasmid for the generation of Sip6-3xFLAG allele at the normal chromosomal location	This study
pSC1509	pKNG101-derived allelic exchange plasmid for the generation of Sip6-3xFLAG allele at the normal chromosomal location of strains harbouring Ssp6-HA	This study
pSC2553	pKNG101-derived allelic exchange plasmid for the generation of chromosomal in-frame Δ*SMDB11_0810* deletion	This study
pSC2554	pKNG101-derived allelic exchange plasmid for the generation of chromosomal in-frame Δ*SMDB11_0810* Δ*SMD11_0809* deletion	This study
pSC1706	pKNG101-derived allelic exchange plasmid for the generation of chromosomal Δ*lacZ*::P_{T5}-*mCherry*-kn^R	This study
pSC2575	pKNG101-derived allelic exchange plasmid for the generation of chromosomal in-frame Δ*ssp6* deletion in strains harbouring Sip6-FLAG	This study

Supplementary Table 2. Oligonucleotide primers and additional details for plasmid construction

Plasmid	Sequence of relevant primers (5'-3')[a]	Description
pSC1264	TATATCTAGAGGGATCAGTTCGATGTGCG	Forward primer to clone upstream region of SMDB11_4673 in pKNG101 (*Xba*I)
	TATAAAGCTTACCTTTTGCCATGACTCAATTCC	Reverse primer to clone upstream region of SMDB11_4673 in pKNG101 (*Hin*dIII)
	TATAAAGCTTTTTGAAAAGGTGTCGAAATGAAGG	Forward primer to clone downstream region of SMDB11_4673 in pKNG101 (*Hin*dIII)
	TATAGGGCCCCGACGACATCAAGTACCTGTTCG	Reverse primer to clone downstream region of SMDB11_4673 in pKNG101 (*Apa*I)
pSC1266	TATATCTAGAGGGATCAGTTCGATGTGCG	Forward primer to clone upstream region of SMDB11_4673 and SMDB11_4672A in pKNG101 (*Xba*I)
	TATAAAGCTTACCTTTTGCCATGACTCAATTCC	Reverse primer to clone upstream region of SMDB11_4673 and SMDB11_4672A in pKNG101 (*Hin*dIII)
	TATAAAGCTTGGCGTTTAGAGCAAGCGTAGAG	Forward primer to clone downstream region of SMDB11_4673 and SMDB11_4672A in pKNG101 (*Hin*dIII)
	TATAGGGCCCGCAGCGCAAGAAAGACGG	Reverse primer to clone downstream region of SMDB11_4673 and SMDB11_4672A in pKNG101 (*Apa*I)
pSC1290	TATATCTAGACCAAGAGATGGCGAACGC	Forward primer to amplify SMDB11_4672A for generation of Sip6-3xFLAG fusion construct by overlap PCR (*Xba*I)
	CACCGTCATGGTCTTTGTAGTCAACGCCATCATGGAACTTC	Reverse primer to amplify SMDB11_4672A for generation of Sip6-3xFLAG fusion construct by overlap PCR
	GAAGTTCCATGATGGCGTTGACTACAAAGACCATGACGGTG	Forward primer to amplify 3x-FLAG tag (see below) for generation of Sip6-3xFLAG fusion construct by overlap PCR
	GAACGCTCTACGCTTGCTCTACTTGTCATCGTCATCCTTGTAATC	Reverse primer to amplify 3x-FLAG tag for generation of Sip6-3xFLAG fusion construct by overlap PCR
	GATTACAAGGATGACGATGACAAGTAGAGCAAGCGTAGAGCGTTC	Forward primer to amplify SMDB11_4672A downstream region for generation of Sip6-3xFLAG fusion construct by overlap PCR
	TATAGGGCCCGCAGCGCAAGAAAGACGG	Reverse primer to amplify SMDB11_4672A downstream region for generation of Sip6-3xFLAG fusion construct by overlap PCR (*Apa*I)
pSC1509	TATATCTAGAGTCGATGTAGATAAAGTCAAAAGATG	Forward primer to amplify SMDB11_4672A for generation of Sip6-3xFLAG fusion construct by overlap PCR (*Xba*I), to incorporate 3xFLAG tag in strains harbouring Ssp6-HA
	Other primers as pSC1290	
pSC2553	TATATCTAGACACGCGCTTCGGCGGTATAAC	Forward primer to clone upstream region of SMDB11_0810 in pKNG101 (*Xba*I)
	TATAAAGCTTGGCATCCGCCATGTCGT	Reverse primer to clone upstream region of SMDB11_0810 in pKNG101 (*Hin*dIII)
	TATAAAGCTTTTTTGCAAAGGTTAACGTATGAAGAAC	Forward primer to clone downstream region of SMDB11_0810 in pKNG101 (*Hin*dIII)
	TATAGTCGACCAGCGATCATGGCTATGC	Reverse primer to clone downstream region of SMDB11_0810 in pKNG101 (*Sal*I)
pSC2554	TATATCTAGACACGCGCTTCGGCGGTATAAC	Forward primer to clone upstream region of SMDB11_0810 and SMDB11_0809 in pKNG101 (*Xba*I)
	TATAAAGCTTGGCATCCGCCATGTCGT	Reverse primer to clone upstream region of SMDB11_0810 and SMDB11_0809 in pKNG101 (*Hin*dIII)
	TATAAAGCTTGATTAAATAACATTGTGTTC	Forward primer to clone downstream region of SMDB11_0810 and SMDB11_0809 in pKNG101 (*Hin*dIII)

	TATAGTCGACCAACGGCTATCAAAAGGTC	Reverse primer to clone downstream region of SMDB11_0810 and SMDB11_0809 in pKNG101 (*Apa*I)
pSC1270	TATAGGCGCCATGGCAAAAGGTGCGAAGG	Forward primer to clone SMDB11_4673 in pNIFTY-MBP (*Kas*I)
	TATAAAGCTTCATTTCGACACCTTTTCAAAAAATAGC	Reverse primer to clone SMDB11_4673 in pNIFTY-MBP (*Hin*dIII)
pSC1273	TATAGGATCCATGAAGGTTTTTTCAGTGCTCATATCAAG	Forward primer to clone SMDB11_4672A in pSUPROM (*Bam*HI)
	TATAGTCGACTTATGCATAATCAGGAACATCATAAGGATAAACGCCATCATGGAACTTCATG	Reverse primer to clone SMDB11_4672A in pSUPROM, incorporating a C-terminal HA tag (*SalI*)
pSC1256	TATAGAATTCTCCAACGCCCCCTACG	Forward primer to clone SMDB11_4673, including RBS, and SMDB11_4672A in pBAD18-Kn (*Eco*RI)
	TATATCTAGAGTGAACTGAAAGCCTTCCGG	Reverse primer to clone SMDB11_4673 and SMDB11_4672A in pBAD18-Kn (*Xba*I)
pSC1271	TATATCTAGAGCAAAAGGTGCGAAGGAAATC	Forward primer to clone SMDB11_4673 and SMDB11_4672A into pSC1236 for fusion of SMDB11_4673 with $OmpA_{sp}$ (*Xba*I)
	TATAGTCGACTGCTCTAAACGCCATCATGG	Reverse primer to clone SMDB11_4673 and SMDB11_4672A in pSC1236 (*Sal*I)
pSC1587	TATAGTCGACGAAAAGGTGTCGAAATGAAGGTTTTTTCAGTGCTC	Forward primer to clone SMDB11_4672A into pSC1597 (*Sal*I)
	TATAGCATGCCTAAACGCCATCATGGAACTTC	Reverse primer to clone SMDB11_4672A into pSC1597 (*SphII*)
pSC1597	TATATGTAGAATGAAAATCGAAGAAGGTAAACTG	Forward primer to amplify SMDB11_4673 incorporating a N-terminal MBP tag from pSC1270, and clone into pSC1236 (*Xba*I)
	TATAGTCGACTCATTTCGACACCTTTTCAAAAAATAGC	Reverse primer to amplify SMDB11_4673 incorporating a N-terminal MBP tag from pSC1270, and clone into pSC1236 (*Sal*I)
pSC2549	TATATCTAGAGCGGATGCCGTAAACATTGACAGC	Forward primer to clone $OmpA_{sp}$SMDB11_0810 in pBAD18-Kn (*Xba*I)
	TATAGTCGACTTATGCATAATCAGGAACATCATAAGGATAAACGCCATCATGGAACTTCATG	Reverse primer to clone $OmpA_{sp}$SMDB11_0810 in pBAD18-Kn (*Sal*I)
pSC2561	TATAGTCGACAGAGGACGTTAAAATGAAGAACTTTATGCTG	Forward primer to clone SMDB11_0809 incorporating RBS from SMDB11_2264 in pSC2549 (*Sal*I)
	TATAGTCGACTCATACGTTAACCTTTGC	Reverse primer to clone SMDB11_0809 incorporating RBS from SMDB11_2264 in pSC2549 (*Pst*I)

Plasmid	**Relevant information**
pSC1290 & pSC1509	Nucleotide sequence of 3xFLAG tag: GACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGATTACAAGGATGACGATGACAAGTAG
pSC2575	The same primers were used as for pSC1264 but the template was genomic DNA from the Sip6-FLAG strain
pSC1706	Synthetic insert produced by Invitrogen GeneArt (ThermoFisher) containing the mCherry sequence from pmCherry-N1 and used to replace P_{T5}-*gfpmut2* with P_{T5}-*mCherry* (*Bam*HI and *Sal*I) in pSAN72[3]
pSC1311	Synthetic insert produced by Invitrogen GeneArt (ThermoFisher): Contains sequence encoding the final 206 amino acids of SMDB11_4673 (not including the stop codon) immediately followed by an HA tag (YPYDVPDYA) then the final five amino acids and stop codon of SMDB11_4673 (repeated to preserve translation of the overlapping downstream gene) and then the following 596 nucleotides downstream of SMDB11_4673 (*Xba*I-*Apa*I)

[a]Incorporated restriction sites for cloning into the respective vector are underlined.

Supplementary References

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