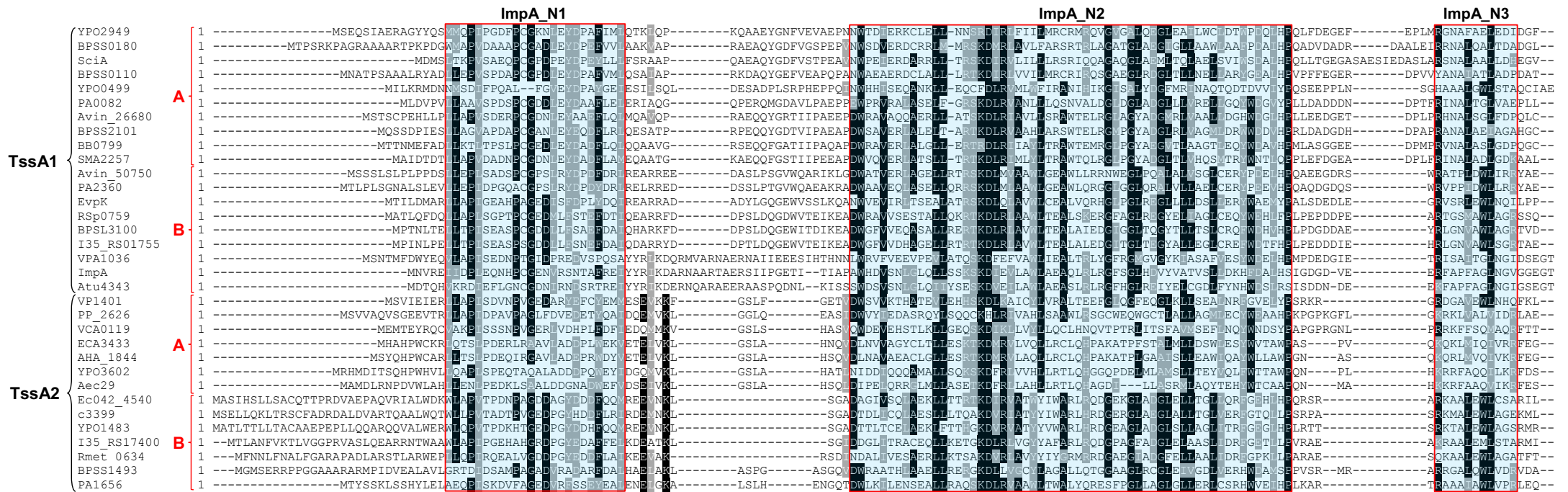


## Supplementary Information

Structural insights into the function of type VI secretion system TssA subunits

Dix *et al.*



**Supplementary Figure 1. Alignment of the ImpA\_N region of TssA subunits.** The N-terminal region of representative TssA1 and TssA2 subunits, corresponding to M1-E136 of *B. cenocepacia* TssA1<sup>B</sup> (I35\_RS01755), were aligned using Clustal Omega<sup>1</sup> and conserved residues were highlighted using Boxshade. Amino acids in white font with black shading are identical at the corresponding position in  $\geq 50\%$  of sequences aligned, whereas those highlighted in gray are similar. The three conserved motifs (ImpA\_N1-ImpA\_N3) that define the ImpA\_N region are enclosed in red boxes with blue coloured fill. Members of the A and B sub-clades of each TssA family are indicated on the left of the alignment in red font. Gaps have been removed between non-alignable amino acids N-terminal to the ImpA\_N1 motif to accommodate TssA sequences in a single line.

a

|  |             |     |                                                                           |
|--|-------------|-----|---------------------------------------------------------------------------|
|  | YPO2949     | 332 | IQNRRAALARLIEVRENFETAMEPSSPVIALAFSEITIGKSFGELIQFPOILLISK--NEGQE-----      |
|  | BPSS0180    | 320 | PVDRYAARELRQARQNFQHEPSSPPIILIRRAEHVGKRYADVQAPALLAL--WSADET-----           |
|  | SciA        | 291 | IRDRNDALERLRIRRFESSEPPSPITPLRQAEHLVGKRFSEVINIPVLLLEK--WDALF-----          |
|  | BPSS0110    | 295 | PMDRWGAALAAQATRLNFEQNEPSSPVIVLROSERMVGKRFSEANAPALLAQ--WDALDV-----         |
|  | YPO499      | 269 | IRSRQETIMMLDRILEYFQHYEPESHAPIFIRRTKEMIGDFYSVEELIPAVIT--KQFTCKTNLFFR---    |
|  | BPSS0515    | 246 | LRSRQEVGAALDRIVEYFVHEPESHAPIFISRIQRLMGAGFEEMALYPTAASI--VAQSRPQSSK----     |
|  | PA0082      | 279 | IANREDVIRQLDRLEBYVVRHEPSSPVVILKRAKTLVTADFAELVRNLIPDGISQ--FETLRPESE----    |
|  | XOO2886     | 276 | VEHPDEVRRLDQICDYARREPPSSPVILLRRAQRLVGDFDTAIRRLAPRAIEE--QVVSQGEHSD----     |
|  | XCV4202     | 281 | IAGPEVKKRLEEICAYYARHEPSSPVEMILRRAQRLVGCDFLAMKLIAPAGIDE--QRVTEPLEET----    |
|  | Daci_3848   | 276 | ARNREQAQLLELAAEVERTEPSNPAILLRRAAREMRMGFMOTILRLISPSLAQ--VEVITGAHLQEQQT--   |
|  | Avin_26680  | 279 | LDSDRVNLLLEKICVYLERHEPESHAPILIRRAQRLMQGFYDILRLAPASLAQ--DLDLGKAPS-----     |
|  | BPSS2101    | 294 | LTSRDDVQLGLEKVCRYFELHEPESHAPILIRRAQRLLSDFYELIRLAPSLPK--DLSGCRNE-----      |
|  | BB0799      | 281 | IATREDAAMLAKVSAYFETHEPESHAPYILRRVQOLIPDFHDIILRLNLAPOGLAQ--FEAWTREAASQS--- |
|  | SMA2257     | 274 | IQSRDDAQLMLDKVKNYFLEHEPESHAPILMDRVQRLITDFMQHVRRLAPGLNQ--ETILGRPDNEENS--   |
|  | N454_00632  | 276 | LNSRAEAQLMLEKVKQYFSQHEPESHAPILMDRVQRLIEIDFMOILRLAPGVHQ--ENIFCRRD-----     |
|  | Avin_50750  | 297 | PGSRDAQYRQLGLIADYLARTEPHSPVYILIRAMEWGDKPLGDLISLISADPEARRWTLIGILK----      |
|  | Pf101_3401  | 297 | PASREFAVRQLLLIABYLARTEPHSPVYILIRREVEWGNKPLSEILGLISADAESRRWTLIGVL-----     |
|  | PA2360      | 300 | PTSRDAYRQLLLIADYLARTEPHSPVYILIRRAVEWGNKPLSEILINADSEARRWSLIGVLP-----       |
|  | Csa1_2257   | 300 | PQSRDEAFEMLNGVARYFIDREPHSPVYILIRRAVWGRNPLEEWLKVIKDHGVIDNRDTIGTQPKDDDY--   |
|  | Psyr_4966   | 295 | PTTRDEAFTMLAGIAQYFQSEPHSPVYILIRAIWGNMPLEGWLNQVICKDSNVVDSIRDVIGTKPEK----   |
|  | PP3088      | 296 | PLTRDEAFTMLAGIAQYFQSEPHSPVYILIRAIWGNMPLEGWLSDVICKNNVVDNRDVIGTR-----       |
|  | F952_02433  | 296 | IQNRQAARVLQDISDYFQANEPHSPVSYMIQRTIIMSQMLPHEWLAQVIKDEHPIQLVQEALGVQPKNEYE-- |
|  | EvpK        | 288 | PRTRDEAARQLSGIAAFETEPHSPVSYIIRDAARWGNMRLDDEWIREVVEDGTRRMKGVLCYDEE-----    |
|  | CV3963      | 293 | IRSRQEAALTEVARYFHHNEPHSPVALLABRAARWAEMSLEEWLQHVKPSGTLSQLQELLIDVRQGD----   |
|  | XOO3514     | 283 | PTIRKEAALQRLQVAEETFEPEHSPVAYLABRAARWGMPLHVWLKRVIKGGSVLNQEEMLDVNQNLQ---    |
|  | Daro_2185   | 293 | IRSRDQAARQLQEIQAETFEPEHSPVAYLABRAAWGTNPLHEWLRIVVKDDSAALLRVEELLGVDPNTGESN- |
|  | RSp0759     | 286 | IRTRAQAALNQLREVAEETFEPEHSPVAYLAARAAWGDMPHLAWLRAVVKDDATLSQEEELGLGDSFADDAS  |
|  | BPS13100    | 303 | IQNRQAQAEQLRAVAKYFEGHEPHSPVAYLADRAAEWADMPHLQWLASVVKDDGSLAHRELIGLKPDDNA--- |
|  | I35_RS01755 | 303 | IQNRQAQADQLRAVARYFQTEPHSPVAYLADRAAEWADMPHLKWLSEVVKDDGSLSHRELIGVRPDEQS---  |
|  | VPA1036     | 303 | MQSRQAALQQLQHVADFESEPHSPVSYISEQIIRWCGMPLPEILAILISDGDAKRSYFRVNGIAEQNEN---  |
|  | BPRA0665    | 305 | LKTRQEAALVQLKHISVEETKEPHSPSYAIDQVVRWSDALPDILAILIDDGSARQGYFRVNGISENDTAS-   |
|  | HCH_04246   | 306 | FRSRDAALNHLVVAEYFETKEPHSPICGALBRAASWGRMSIQENLMILIPDEEARARYALMTGIGIGEDAAPI |
|  | ImpA        | 286 | IRSRDEAFELLIAVARYFETKEPHSPSMSETLVRGRMDFFELAILLPQQOTRNAVLTAAIGIQPVADKGG-   |
|  | Atu4343     | 278 | ISSRDEAFETLLSVARYFETKEPHSPSLSETLVRGRMDFSEILAILLPQQARNVLTAAIGIKPGGDNNKG    |

EPxxP

b

Nt2

TssA2<sup>A</sup>

|             |     |                                                                                                                             |
|-------------|-----|-----------------------------------------------------------------------------------------------------------------------------|
| PP2626      | 224 | EREETRAI---LSMAELINODPYDATGQLRRFGWAHNOVAEQVRQG-NITELMA-VPLNITRFVEEAKAVDPALLQRLEKSLT--ACFWIRGSFLAATVATQLMC--EVVEAIRCATA      |
| VCA0119     | 218 | DHAARQTL---LKVADFTAEQEFGIALSIRLRRAHWGSLITSFPHKPK-GETTLRG-VQDRVKDYQDQIR--HPLDALWRKVEOSLT--MAPWFFGQNM SYTIAQQLCKS--DWCQIAEETQ |
| ECA3433     | 223 | ERSWRQTO---LNVAALIVERHPDSPMGYRLRRHAWSGATPEMSSKG-HNTQLAP-VSSERVDEYQSA--QADLAWERVEOSLV--MAPWFDGHMSASVASLCHS--SVATAAEBLS       |
| AHA1844     | 231 | DRAMRQTO---LKVAELLIERQPEVAVGYRLRRHAWGHTAFPMSGAG-NITPLAP-VSADMVDEYIAAN--APFQGLWQRVEOSLT--MAPWFGHRSAEVAELGFG--AVAQATAEBLG     |
| YPO3602     | 220 | DRAMRQTL---LKVAADLSEQOPDAAIGHLRRHAWGATAPPMQAQS-GRTEPLAA-VSADRTADVLARA--NADPEWHQVEOSLT--MAPWLDGHVISAQIALQLGYD--AVAQAIRDELS   |
| Aec29       | 233 | DRAMRDTL---LKVAAILCEBPQESPQCHRLRRHAWQITSTPQAES-GRTEPLAA-FPVDMMDDYLARN--NADPEWHQVEKSL--MAPWLDGHVISAQTALQLCYK--QVADAIRDEVT    |
| Ec042_4540  | 223 | ITSGQELLSQAIVLAKYRTOPEGWLAHRLMSVRHDTTHQIPPLSAG-GRTRTAP-PGPDRRASLRILYLOON-WLSLLEQCDMFARGASHLWLDLQYIHQALLQTK--ENYAATIQYDLK    |
| c3399       | 226 | ITSGRDLDDQAIVLARYNEQOPQGWLSHRLMTILRMDTTHEPP-DV-LGITRLAP-PRTESRNQLRLYLAQON-WTELLQALIMFSTGSHWLDLQYILHQALTACVPWDRWTAVIRODIA    |
| YPO1483     | 229 | VQSGRDLDDQTILAKYRNOPQGWLSHRLMSVRHDTVHQSPPLDVN-GRTRTAP-PRPVRVQLRLYLOON-WLSLLEQAEISFAEGNHWLWLDVQYILHQALSACAPFDGWASCTQDLR      |
| I35_RS17400 | 217 | IASARDVLDQAALSAWREGEENGYPSPVRAASVRMDITHPPPSDAA-SHTRITP-PRALLRQQLRLYLKQ-QWHELERVEGAFMEGNHNLWLDLQYFQHVALDNGAPYRAWSEVRVDF      |
| Rmet_0634   | 218 | VTSTRDLDDRAALQALFFRQPDGYLARYLMRCVVRMDITTEAPPEHAS-GRTRTAP-PRALLRAQRLRLYLKQ-WPELIDRIEPAPFAEGANHNLWLDLQYAFMAQDAGGAYARVRLIATLCA |
| BPSS1493    | 330 | ERALADALQALCVATATAQADWADARGHLRLRVACVSCAPETDANGRTIRAA-PSASIVGAASGIDGDGE-PYVAFRAFAHAQ--AFPLWLDLQYLAARALAGGADGARREVEVAVR       |
| PA1656      | 227 | MRAQQAA---LPCAWWLKPKATDLRLRLNRTMILWLPES-IPERNAE-QVDAERG-VPAKLKLSYQERFAGL-YADLIVEEASLA--RAPWFDGQRVWECLQGLNAE--QAMREVMHFA     |

Nt2

TssA2<sup>A</sup>

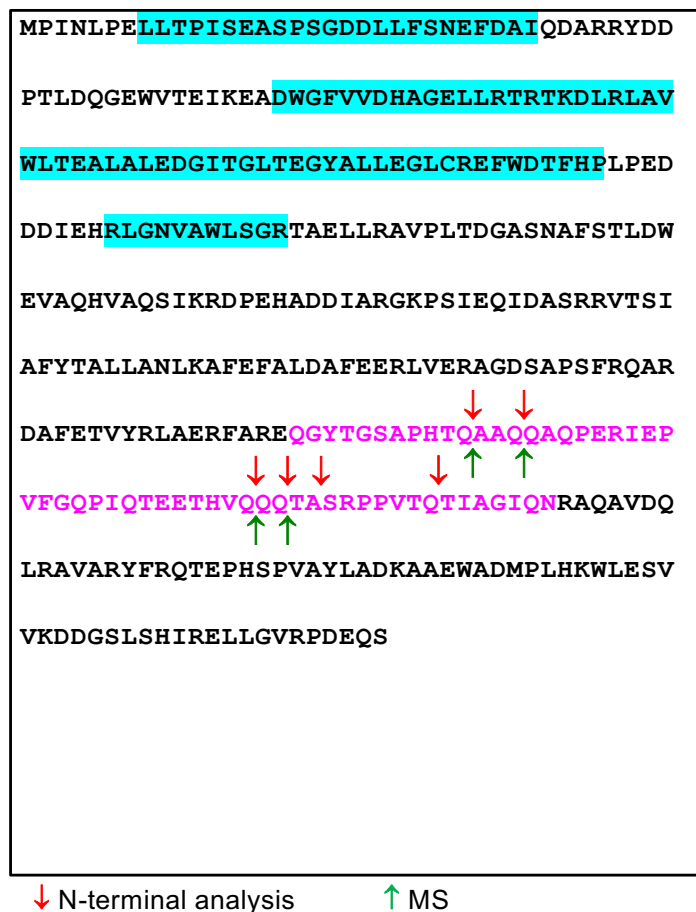
|             |     |                                                                                                                           |
|-------------|-----|---------------------------------------------------------------------------------------------------------------------------|
| PP2626      | 340 | RFVQRNPSIQCCFSDGMVFVDQCLAWLKGTT-(N)25-TGGTEPIILLRLGLQAGVRAPRERCHTTIADDLAARG-SWLAQDLCANVARTMQQTASAWEPFERIQQYASPVLA-VQDKD   |
| VCA0119     | 332 | QFIRRLPSLLEKFRGGEPFVS-SVKBWL-(SV)-(N)27-EGGAYALSMNLNGVSAVEPRDKFYWRLLSADLLRANHDAAGEQYQTLNQVTTLSIPWNEPSLLEQORYTTSE-----     |
| ECA3433     | 337 | AFVQRLPETREKAFSDGAPFLTGKCSQWLSSS-(N)23-EKGGAALLLDERVRLKEPDRRYAEVADLDAEBGKSLAAQHYQHWWQESQQLGMQWEPGYSRVERLAASRK-----        |
| AHA1844     | 345 | TFLQRLPLRLREAFSDGPPFLSPESCRWLQFA-(N)21-EQGAAALALDDEAQLKEPRDRFHALLQABELLAQBGVEALARQHYQHLLQWESASRLGLSHWEPGLNRRESLAAPLS----- |
| YPO3602     | 334 | VFLARPNLKTITFFRDMTPFLSSSAALQMN-ROEQQSEPRDRFYHOLSAOLEKAGTALAQQHYHSLLLVGQQLQISWEPALALITEQRQLKP-----                         |
| Aec29       | 347 | DFLARLPGLINLELNDRTPFVSCHKQWLSSS-(N)24-ENGEEAALRYLE-NIPE-GDPRHQHRHFFGCAQLLEEAGVQLAQQQYRMFRTGLHMLSEWEPSSLKAEQLLTAEQ-----    |
| Ec042_4540  | 343 | GILLRLPGELTAFNDGMPFADVILSWHQQQ-(N)37-NEGEEAALSGLQ-ARFGIQSDRSNLLRLRLVARAEQYGNKNDLHLLABELDERATRIILSQWEPGLFEVKARLKLLMKSAKTE  |
| c3399       | 347 | LLERLPGLENAANDGTPPFADSVTRNWIQQQ-(N)37-SQGEEAALGWLQ-TREGGITTAQRLLRLRLVARAEQYGNKNDLHLLABELDITAAQGTITQWEPGLFEVKARLKLLLRHRH-  |
| YPO1483     | 351 | LLTRLPGLEGTCSGDTPFADSVLQWLNQQ-(N)34-SQGEEAALNWLQ-SREGGITTAHQLLRLRLVARAEQYGNKNDLHLLSGLDSSGALLTPQWEPGLFEVKARLKLLMKAQRGD     |
| I35_RS17400 | 339 | LFLRLPGERISFSDGTPFSDITLLEWIRRH-(N)40-RDGEEAFAWLQ-ALPGMKTGHRHLLRLRLVARALAHAGREDTALLLVLDITSSRSILPMHNEPALAFEVKQQLVRALATGNRKD |
| Rmet_0634   | 340 | LLERLPGLDQAFSDGTPFADATLEWIRARY-(N)36-QQSEEAFAWLQ-GEPAPAGERDRFVROLRLVARAERVDRADTALHLLSALETGTQRYOLANWEPSLAFEVKQLLRLLVRLTRKD |
| BPSS1493    | 451 | ALLARLPGDALTTFADGTPFADATRAWLDEL-(N)58-SGQDLALHATQHA-DRATSABQRLRARLRCEAARDHWPEPEAFARGIEPIRRHDLAWNPELALDGLSAAAYA---LIRRDRE  |
| PA1656      | 342 | LLQRLPGIVETREEDGAEFAAATRWIRSAH-(N)35-KDGKAAVQLKQG-KRAHGGARERFWOISLARCFLAKKYELAKTQLESIDHQLHESGHAMEPELALDHLHLLHSCCELLPQNH   |

TssA2<sup>B</sup>

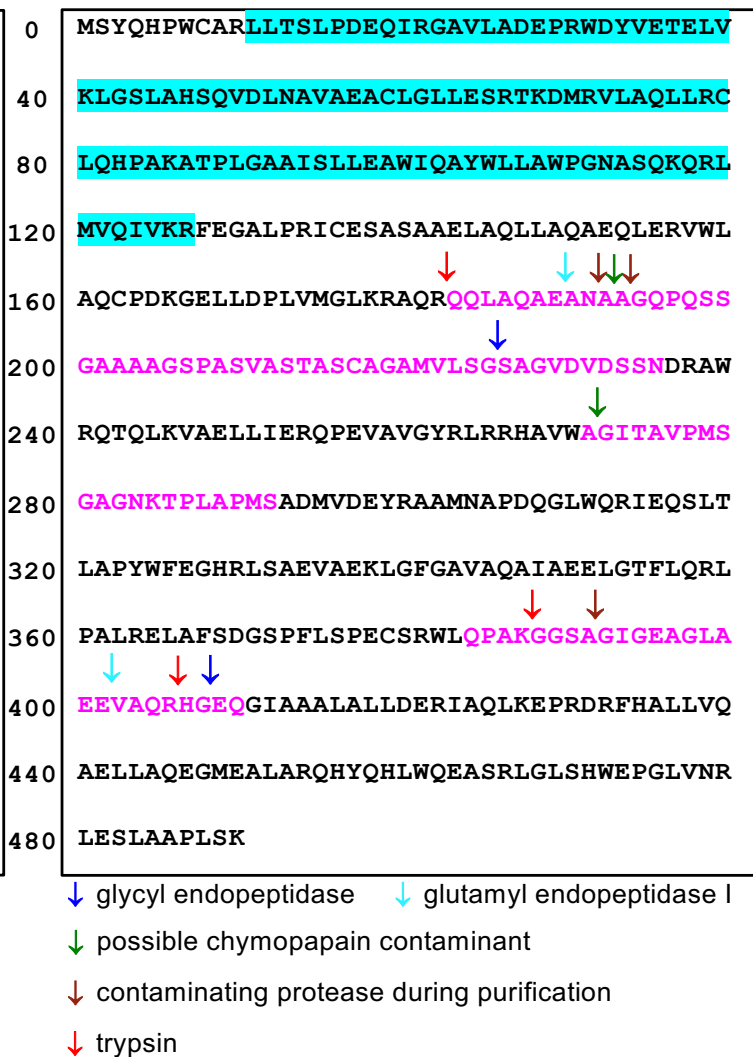
Supplementary  
Figure 2

**Supplementary Figure 2. Alignment of C-terminal amino acid sequences of TssA1 and TssA2 subunits.** Amino acid sequences of the C-terminal regions of representative TssA subunits were aligned using Clustal Omega<sup>1</sup> and conserved residues were highlighted using Boxshade. Amino acids in white font with black shading are identical at the corresponding position in  $\geq 50\%$  of sequences aligned, whereas those highlighted in grey are similar. **(a)** Alignment of TssA1 C-terminal domains. The CTDs of representative TssA1<sup>A</sup> and TssA1<sup>B</sup> orthologues are shown along with the last three residues of the predicted interdomain linker. The location of the conserved EPxxP motif is shown below the alignment. **(b)** Alignment of TssA2 Nt2 and C-terminal domains. Representative members of the A and B sub-clades, based on a phylogenetic analysis of full-length TssA2 subunits, are shown. Sequences shown in magenta are non-conserved and are predicted to constitute an interdomain linker connecting Nt2 to the CTD (the number of linker amino acids that are not shown is indicated). For clarity, the amino acid sequences at the extreme C-terminus of eight orthologues are not shown.

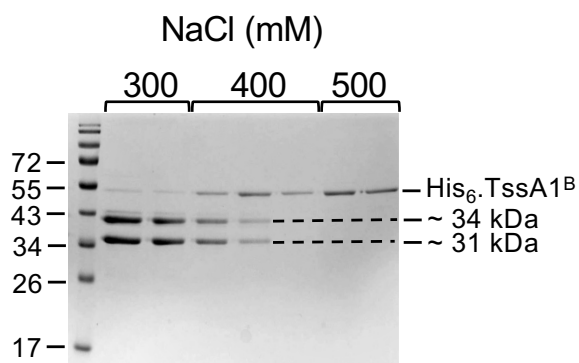
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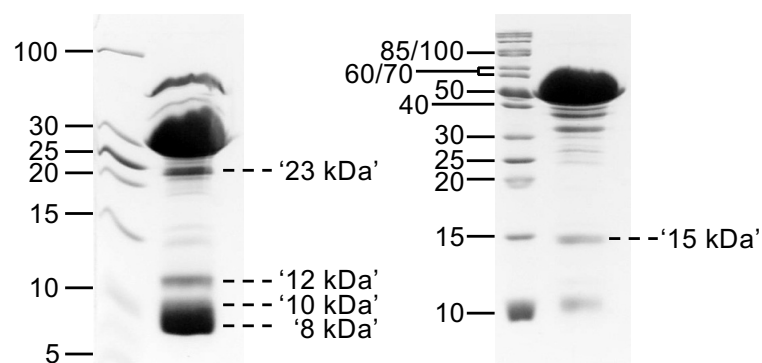
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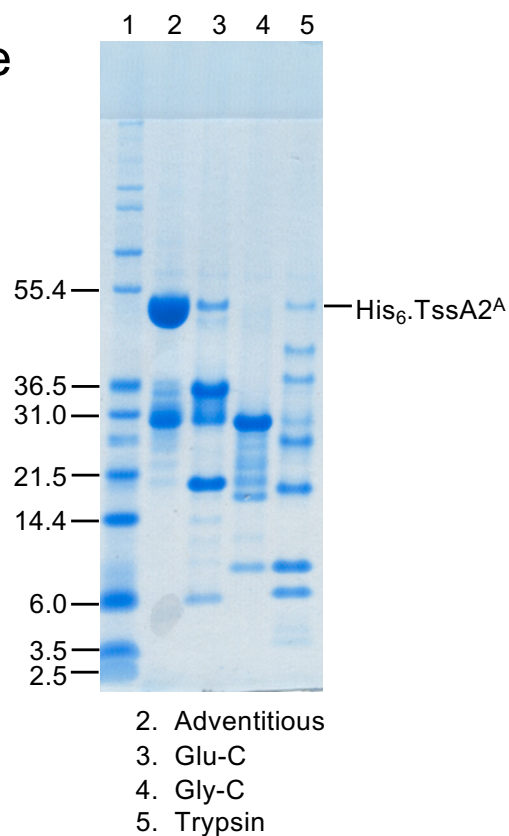
c



d

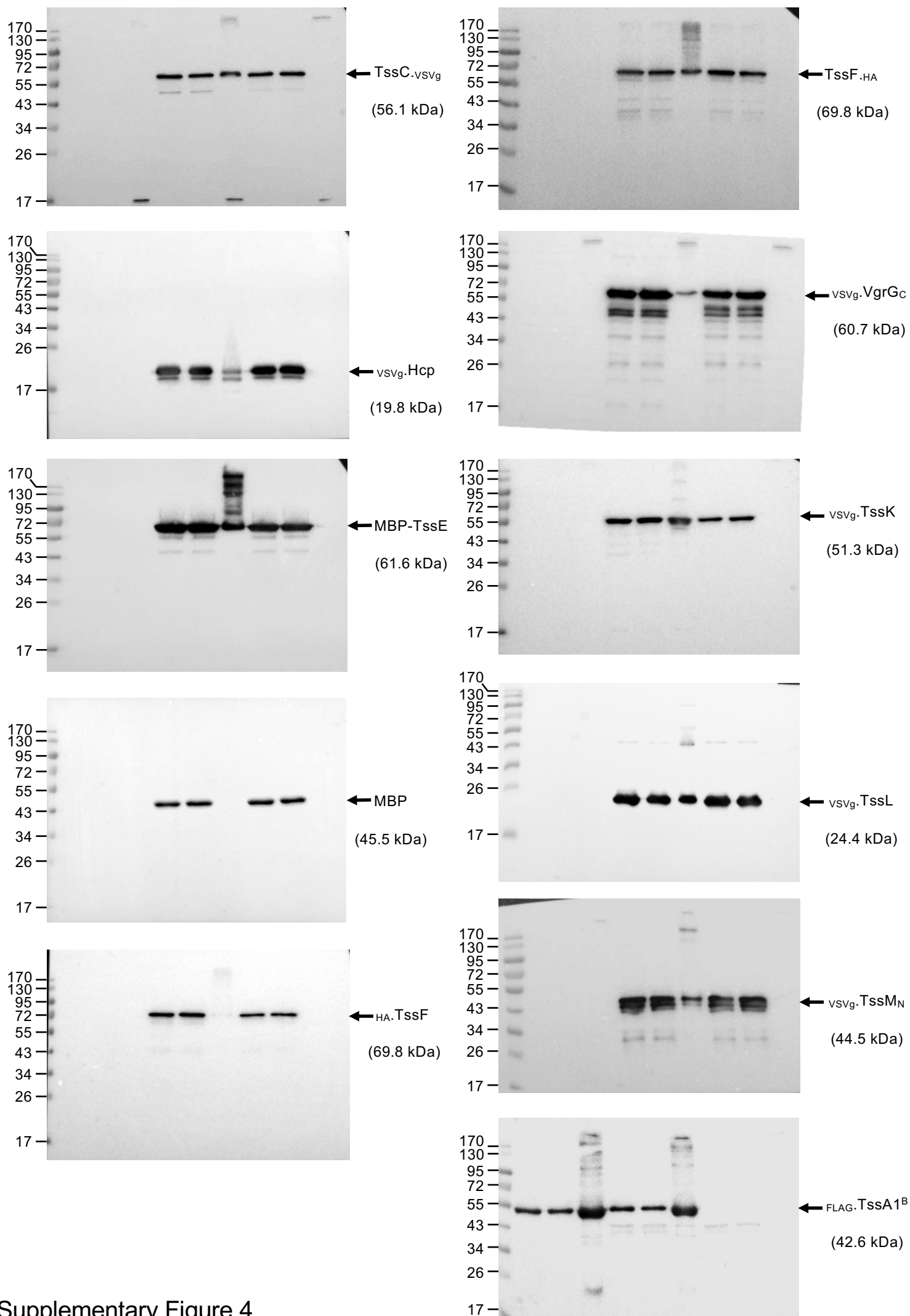


e



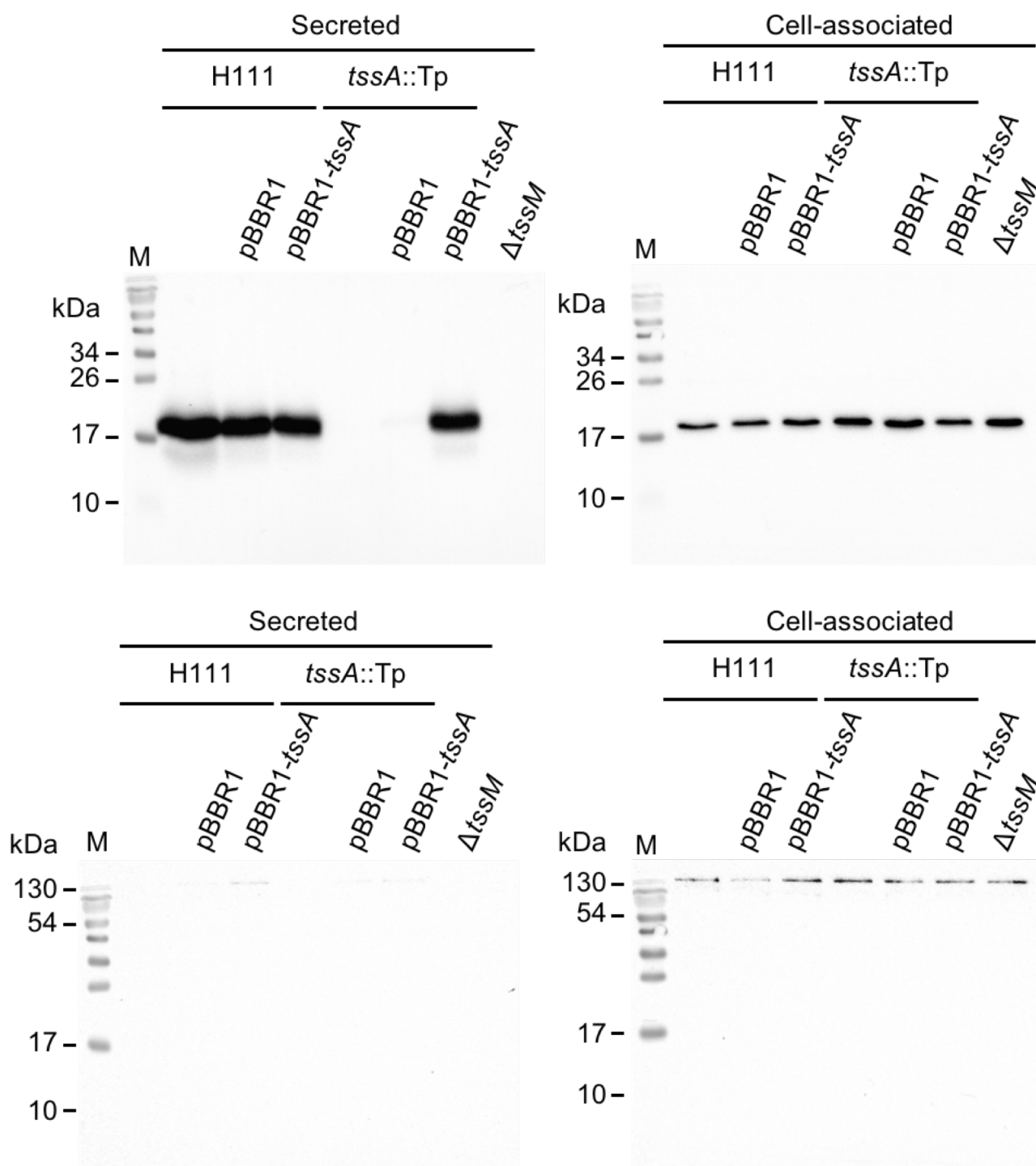
Supplementary Figure 3

**Supplementary Figure 3. Domain analysis of TssA1<sup>B</sup> and TssA2<sup>A</sup>.** Sequences of Bc TssA1<sup>B</sup> (a) and Ah TssA2<sup>A</sup> (b) with interdomain linkers shown in magenta font. (a) Adventitious protease cleavage sites utilised in interdomain linker identification are indicated by up and down arrows, for mass spectrometry and N-terminal analysis, respectively. (b) Arrows indicate protease specific cut sites within Ah TssA2<sup>A</sup> identified by mass spectrometry that were used to identify the interdomain linker. (c) Analysis of large proteolytic degradation products of Bc TssA1<sup>B</sup>. 12% SDS-PAGE analysis of His<sub>6</sub>.TssA1<sup>B</sup> following IMAC purification, dialysis and concentration, and application to a Q sepharose column in 20 mM Tris-HCl (pH 8.0) with elution by a NaCl step gradient, revealed two major species (~31 kDa and ~34 kDa), that corresponded to polypeptides of 31.3, 31.5, 34.2 and 34.4 kDa by mass spectrometry. Protein molecular weight markers are shown in kDa. (d) Analysis of small proteolytic degradation products of Bc TssA1<sup>B</sup>. 15% SDS-PAGE analysis of His<sub>6</sub>.TssA1<sup>B</sup> degradation products before application to the Q column (left hand gel) and following elution (right hand gel), identified a series of low molecular weight peptides ~8, 10, 12, 15 and 23 kDa, these peptides were subjected to N-terminal amino acid sequence analysis. Protein molecular weight markers are shown in kDa. (e) Analysis of Ah TssA2<sup>A</sup> proteolytic digestion products. SDS-PAGE analysis of His<sub>6</sub>.TssA2<sup>A</sup> following proteolytic digestion with contaminating protease (lane 2), glutamyl endopeptidase (Glu-C) (lane 3), glycyl endopeptidase (Gly-C) (lane 4), and trypsin (lane 5). Protein molecular weight markers are shown in kDa.

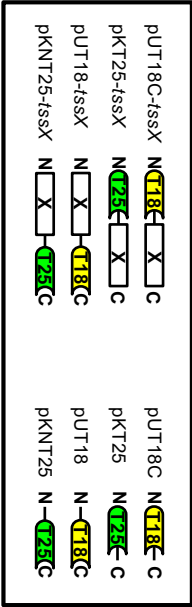
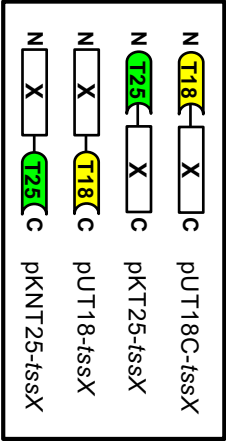
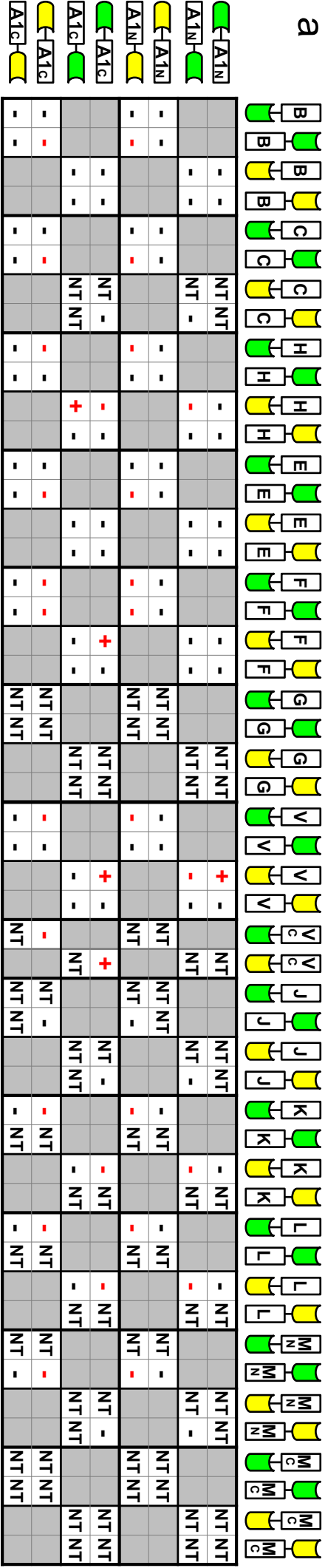


Supplementary Figure 4

**Supplementary Figure 4. Co-immunoprecipitation analysis of FLAG-tagged Bc TssA1<sup>B</sup> with other T6SS subunits.** Uncropped images of western blots presented in **Fig. 2c** are shown. Molecular weights of the detected T6SS subunits (and control MBP) are indicated (which include any epitope, affinity or solubility tags) and markers are indicated in kDa. See legend to **Fig. 2** for more details.

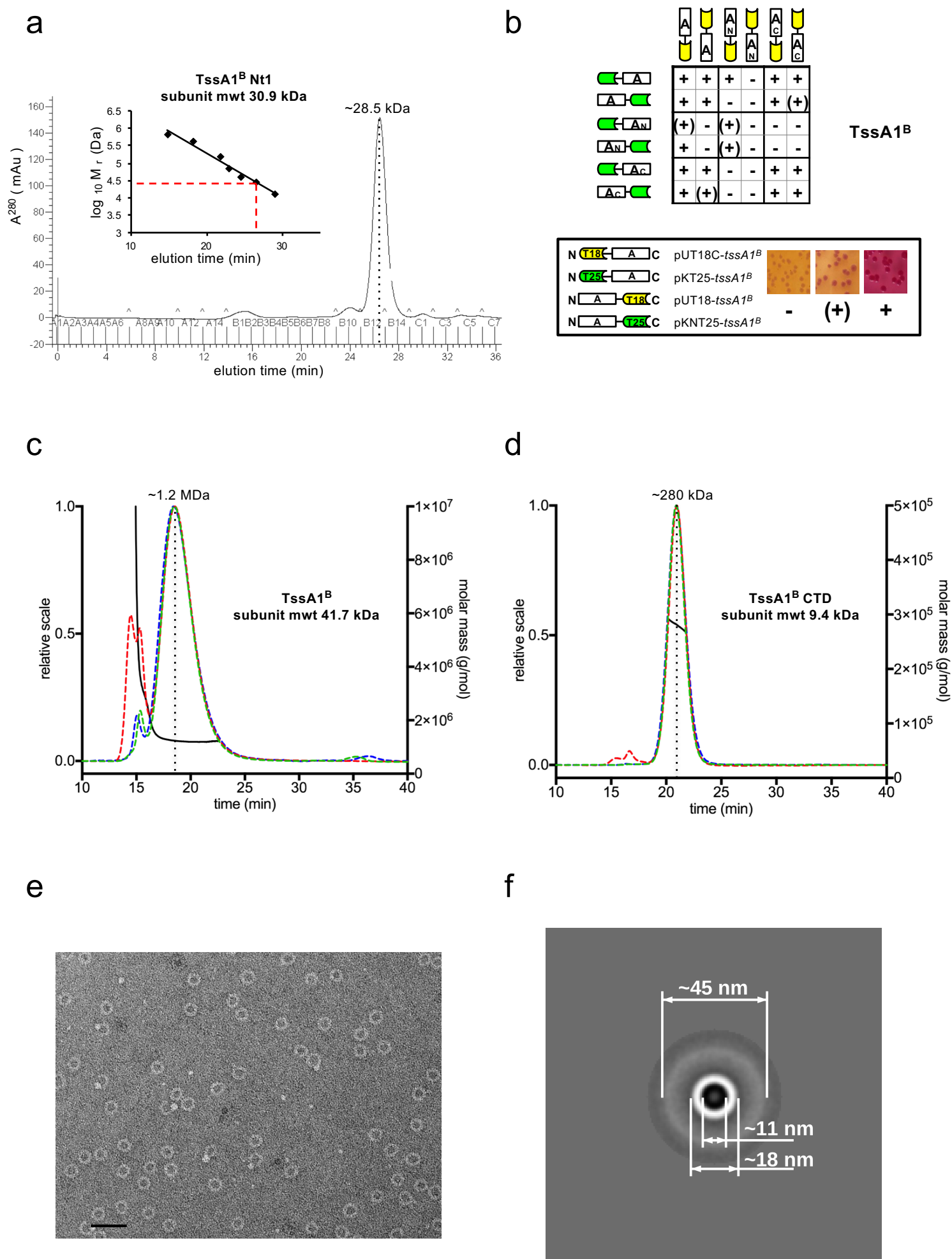


**Supplementary Figure 5. Effect of TssA1<sup>B</sup> inactivation on T6SS secretion activity.** Uncropped images of western blots presented in **Fig. 3** are shown. See legend to **Fig. 3** for more details.



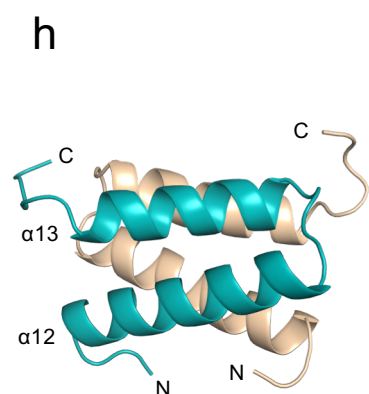
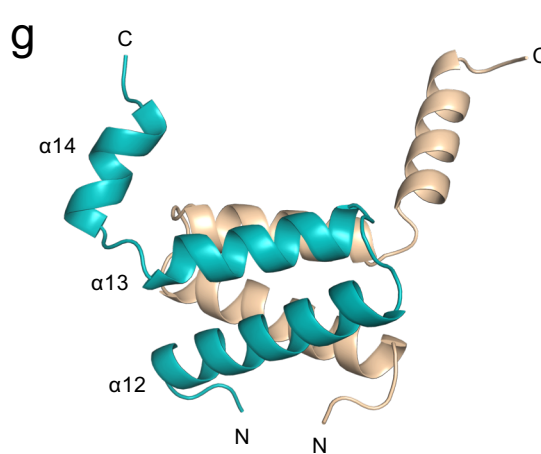
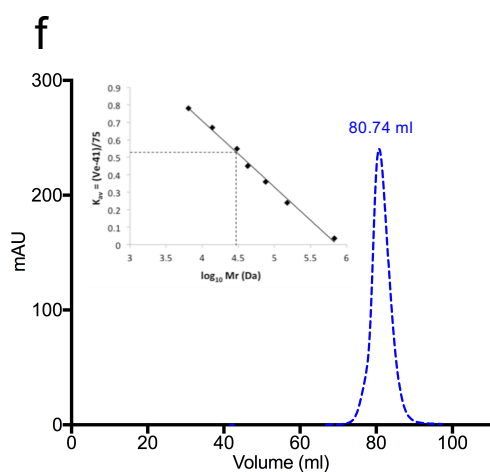
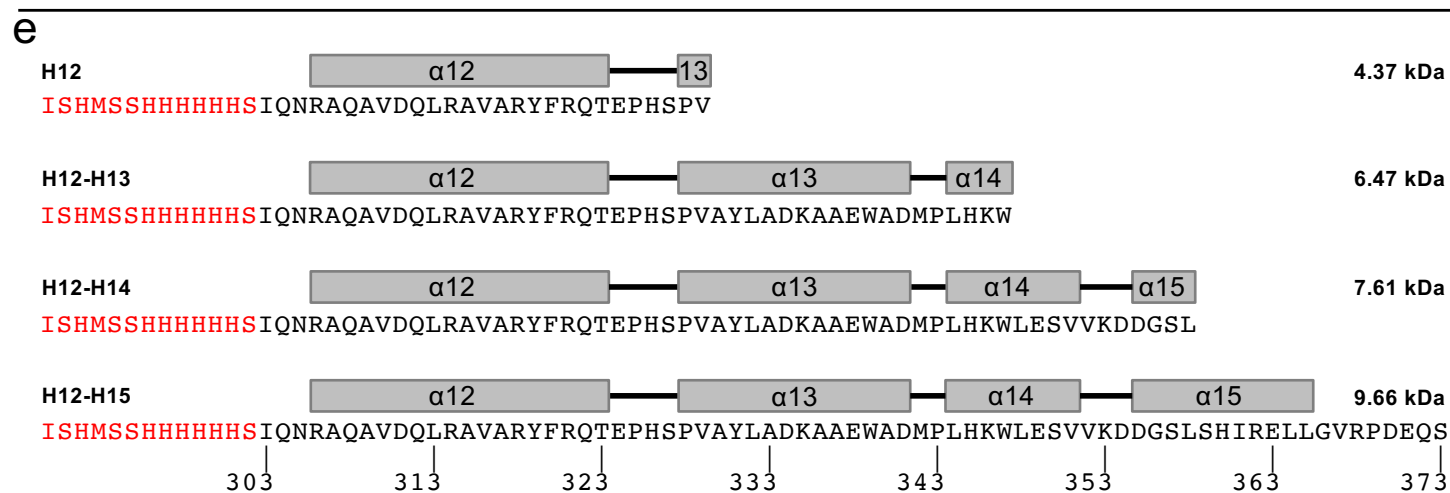
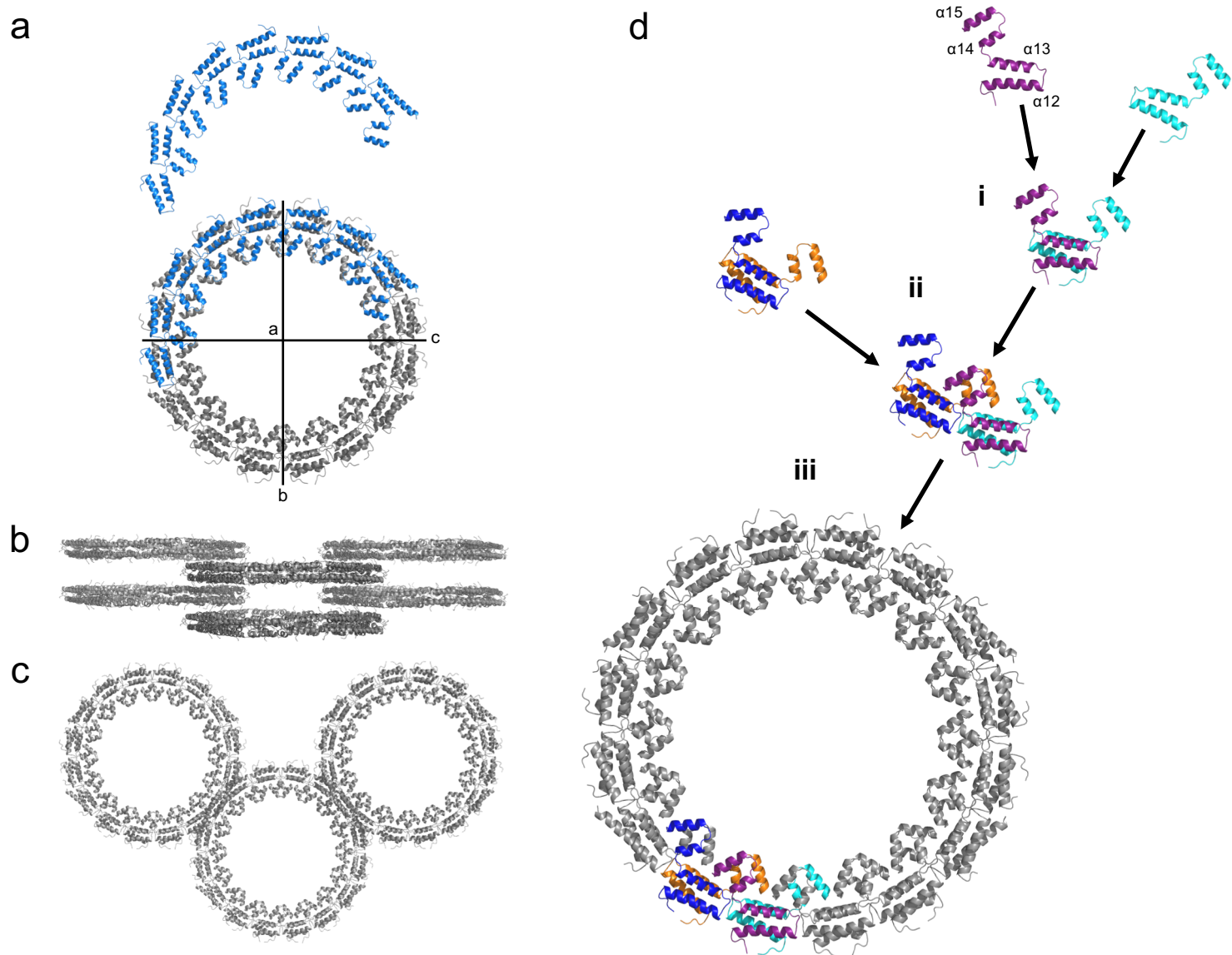
Supplementary Figure 6

**Supplementary Figure 6. Two-hybrid analysis of TssA1<sup>B</sup> domain interactions.** (a) Each hybrid protein is represented by a green or yellow coloured motif indicating the CyaA fragment (T25 and T18, respectively) linked to a white rectangle labelled according to the T6SS subunit or TssA1<sup>B</sup> domain. T6SS subunits are indicated by a single letter corresponding to the suffix used in the Tss nomenclature (i.e. B corresponds to TssB) except for H (Hcp), V (VgrG) and V<sub>C</sub> (the VgrG core region, although only two hybrid proteins were constructed in this case). A1<sub>N</sub> and A1<sub>C</sub> correspond to TssA1<sup>B</sup> NTD (Nt1) and CTD, respectively. Interactions of the cytoplasmic N- and periplasmic C-terminal regions of TssM (M<sub>N</sub> and M<sub>C</sub>, respectively) were investigated separately. Compatible pairwise combinations of BACTH plasmids encoding the indicated hybrid proteins were introduced into *E. coli* BTH101 and the resulting Mal phenotypes were scored on maltose-MacConkey agar. Colonies were recorded as +, positive or -, negative after 120 h incubation at 30 °C. Combinations involving TssG and TssM<sub>C</sub> that gave rise to a maltose-negative phenotype in combination with TssA1<sup>B</sup> were not tested (NT). In most of the other cases only representative combinations were tested. Results shown in red font are from plasmid combinations that were quantitated by  $\beta$ -galactosidase assay and shown in (b). (b)  $\beta$ -galactosidase activities in *E. coli* BTH101 containing representative pairwise combinations of TssA1<sup>B</sup> domain and T6SS subunit hybrid proteins were assayed. A<sub>N</sub>, TssA1<sup>B</sup> Nt1 (N-terminal 256 aa); A<sub>C</sub>, TssA1<sup>B</sup> CTD (C-terminal 80 aa); Z represents the Zip positive control. Otherwise, nomenclature is as in (a). Combinations involving TssG, TssJ and TssM<sub>C</sub> were not assayed as they do not appear to interact with TssA1<sup>B</sup>. Data is representative of three independent experiments (n = 3) performed in duplicate and values correspond to the mean  $\pm$  standard deviation. Values presented in **Supplementary Data 1**.



Supplementary Figure 7

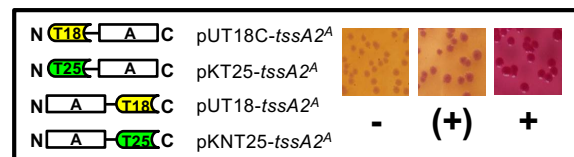
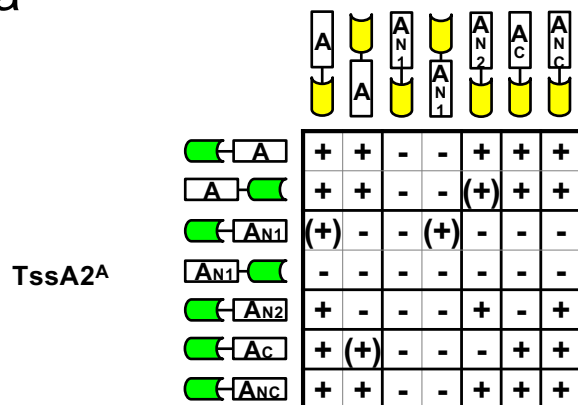
**Supplementary Figure 7. TssA1<sup>B</sup> self-associates into a high molecular weight ring-like oligomer.** (a) SEC profile of the Bc His<sub>6</sub>.TssA1<sup>B</sup> Nt1 domain indicating a monomer of 30.6 kDa is present. (b) Self-interaction analysis of Bc TssA1<sup>B</sup> and its domains. Each hybrid protein is represented by a green or yellow coloured motif representing the CyaA fragment (T25 and T18, respectively) linked to a white rectangle labelled according to the fused region of TssA1<sup>B</sup> (see key). All four possible hybrid proteins were constructed and analysed for TssA1<sup>B</sup> and its domains in pairwise combinations (N, N-terminal domain (Nt1); C, C-terminal domain (CTD)). Colonies were recorded as +, positive; (+), weak positive; or -, negative (see key). (c) SEC-MALLS elution profile, peak positions, and molar mass plot of TssA1<sup>B</sup> (~1.2 MDa). The normalised signals for light scattering (LS) are shown in red, refractive index (RI) in blue, UV absorption (280 nm) in green, and molecular weight estimate curve is shown in black. The molecular weight was determined by a Zimm fit procedure at each point. The X-axis is the elution time in minutes. (d) SEC-MALLS elution profile, peak positions, and molar mass plot of TssA1<sup>B</sup> CTD (~280 kDa). Details as for (c). (e) Negative stain EM of single particle views of TssA1<sup>B</sup> CTD. Scale bar is 50 nm. (f) Negative stain EM average of TssA1<sup>B</sup>. Measurements indicate the inner diameter of the CTD ring (110 Å), the CTD ring outer diameter (180 Å), and diameter of the whole particle (450 Å).



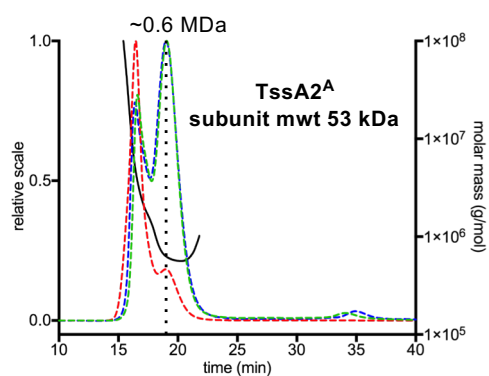
Supplementary Figure 8

**Supplementary Figure 8. Assembly of the TssA1<sup>B</sup> CTD ring oligomer.** (a) Formation of the TssA1<sup>B</sup> (I35\_RS01755) CTD ring from the 8 subunits of the asymmetric unit. The contents of the asymmetric unit (blue) are related to the remaining sections of the ring (grey) by the 2-fold crystallographic symmetry axes around a, b and c. View is down the crystallographic axis a. (b) and (c) Packing of TssA1<sup>B</sup> CTD ring oligomers in the crystal lattice. (b) View perpendicular to the 16-fold axis, (c) view parallel to the 16-fold axis. (d) Proposed assembly pathway for the TssA1<sup>B</sup> CTD ring oligomer. (i) Two monomers (purple and cyan) dimerise, via helices  $\alpha$ 12 and  $\alpha$ 13; (ii) two dimers (purple/cyan and orange/blue) assemble with the majority of interactions occurring between helices  $\alpha$ 14 and  $\alpha$ 15 (purple and orange); (iii) sequential addition of further dimers (or higher order oligomers) results in assembly of a ring containing 32 subunits that exhibits D<sub>16</sub> symmetry. (e) Nested deletion of TssA1<sup>B</sup> CTD, containing an N-terminal MBP.His<sub>6</sub> tag. The sequences shown are released upon Factor Xa cleavage, with remaining His<sub>6</sub>-tag residues highlighted in red. Construct H12-H15 corresponds to full-length TssA1<sup>B</sup> CTD and was shown to form ring oligomers by EM, whereas the truncated derivatives form lower molecular weight complexes. (f) SEC profile of the H12-H14 construct on Superdex 200 (following removal of the MBP tag) to show failure to assemble ring oligomers. The elution peak is seen to shift from a high molecular weight structure ~355 kDa for selenomethionine-incorporated TssA1<sup>B</sup> CTD<sup>2</sup>, to a much lower molecular weight of ~30 kDa for the H12-H14 construct. SEC also showed that the MBP-tagged H12 construct formed monomers while MBP-tagged H12-H13 formed dimers. (g) Crystal structure of H12-H14 determined to 2.35 Å showing dimer formation. Residues 303-358 and 303-354 are resolved in chains A (wheat) and B (teal), respectively. (h) Crystal structure showing dimer formation by a truncated TssA1<sup>B</sup> CTD derivative containing residues 303-347 (corresponding to helices  $\alpha$ 12 and  $\alpha$ 13) that arose from adventitious clipping of TssA1<sup>B</sup> CTD following its release from MBP.TssA1<sup>B</sup> CTD (303-373). Structure determined to 1.78 Å resolution.

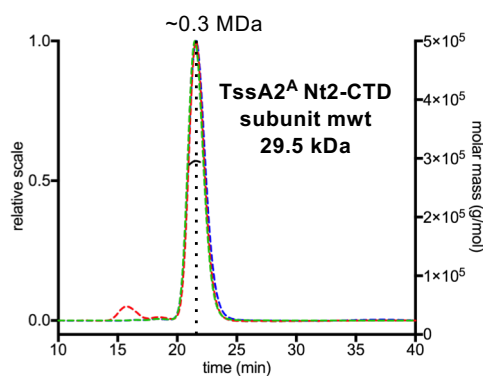
a



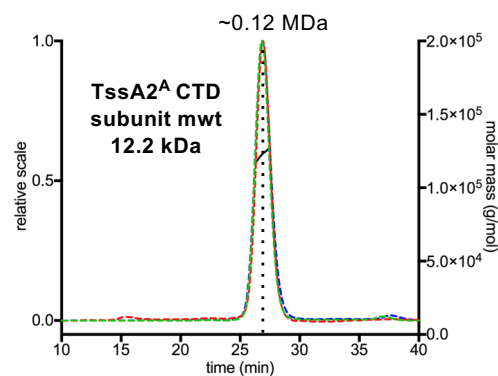
b



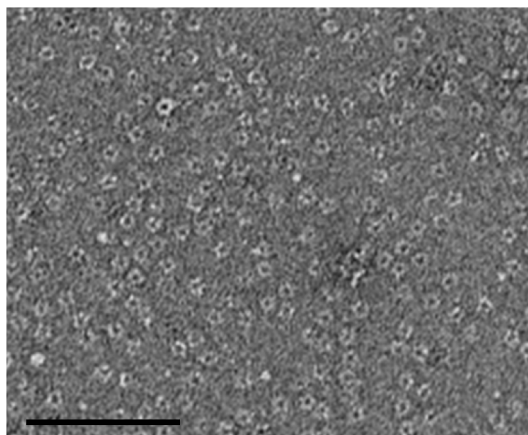
c



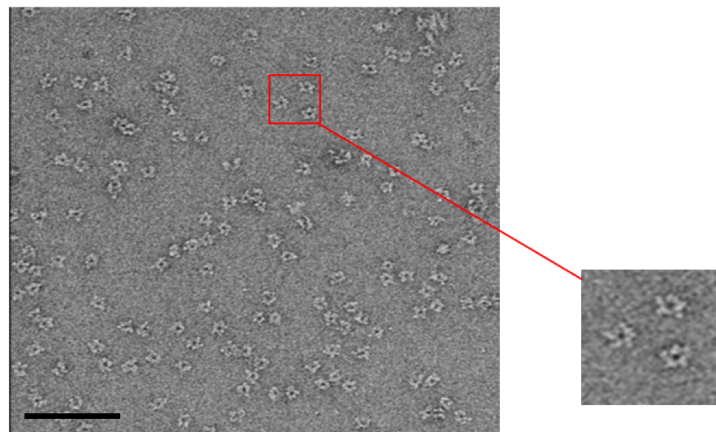
d



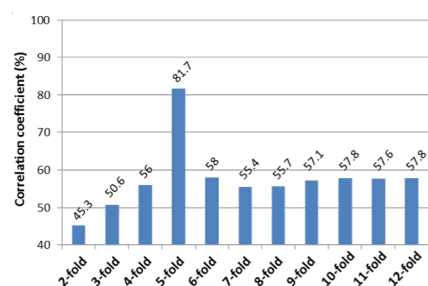
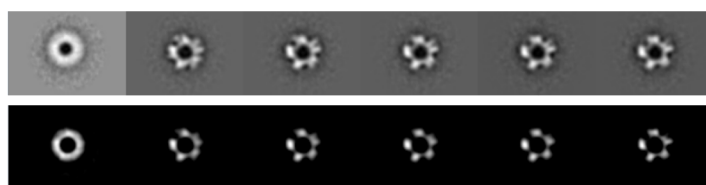
e



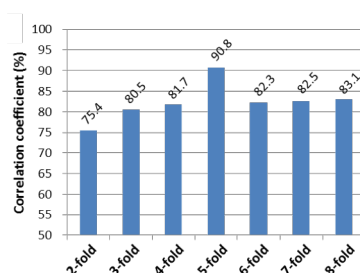
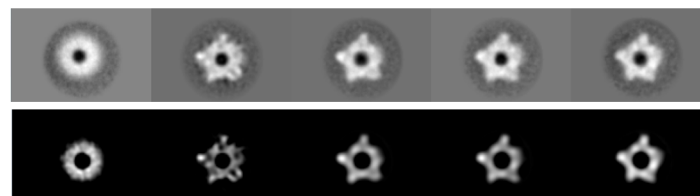
f



g



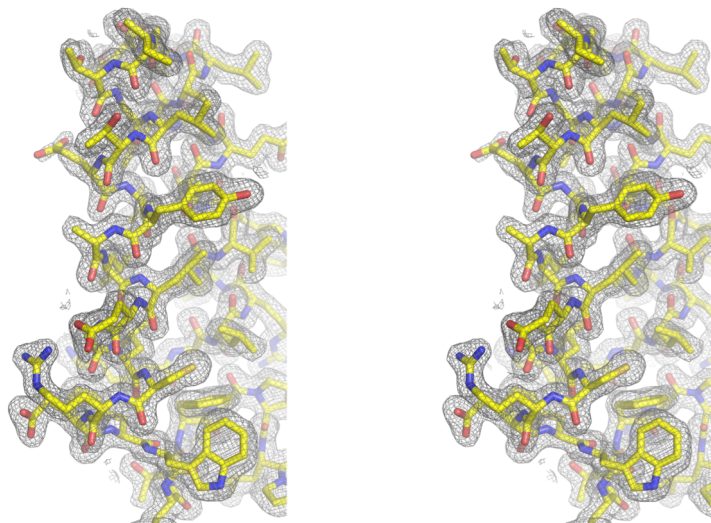
h



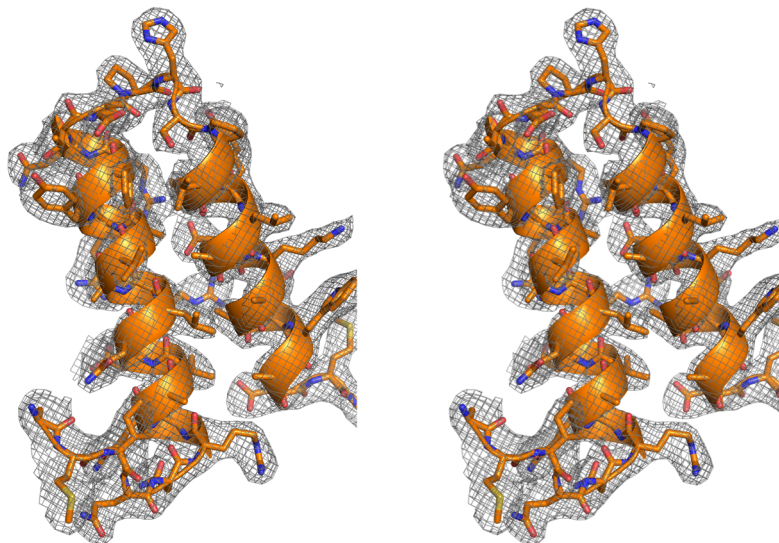
Supplementary Figure 9

**Supplementary Figure 9. TssA2<sup>A</sup> self-associates into a high molecular weight ring-like oligomer.** (a) Self-interaction analysis of Ah TssA2<sup>A</sup> and its domains. Hybrid proteins are represented as in Suppl. Fig. 6b All possible hybrid proteins were constructed and analysed for TssA2<sup>A</sup> and its domains (N1, N-terminal domain (Nt1); N2, middle domain (Nt2); C, C-terminal domain (CTD); NC, Nt2-CTD region). (b)-(d) SEC-MALLS elution profile, peak positions, and molar mass plot of TssA2<sup>A</sup>, TssA2<sup>A</sup> Nt2-CTD and TssA2<sup>A</sup> CTD, respectively. Details as for Supplementary Fig. 7c. (e) Negative stain EM of Ah TssA2<sup>A</sup> CTD single particles. Scale bar is 100 nm. (f) Negative stain EM of Ah TssA2<sup>A</sup> Nt2-CTD single particles, with a sample of individual particles selected in the magnified insert. Scale bar is 100 nm. (g)-(h) Class averaging and correlation co-efficient calculation for Ah TssA2<sup>A</sup> CTD and Ah TssA2<sup>A</sup> Nt2-CTD particles, respectively. The x-axis shows the specific symmetry that was tested, and the y-axis shows the correlation coefficient (%) of all the single particles rotated at a specific angle according to the fold of symmetry, then correlated to their unrotated self.

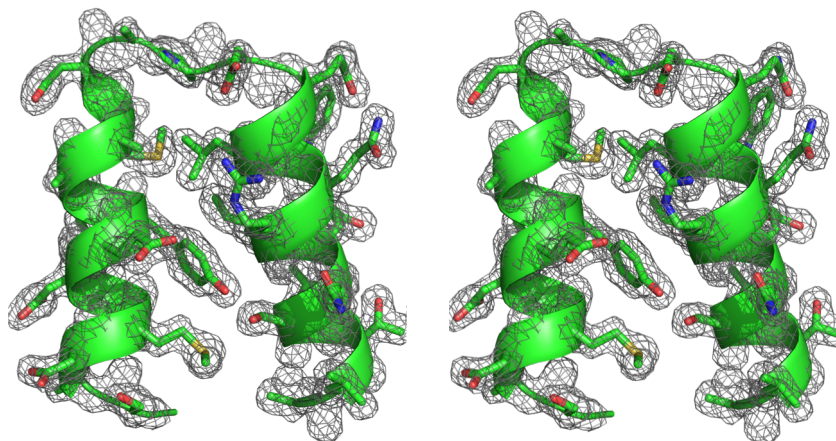
a



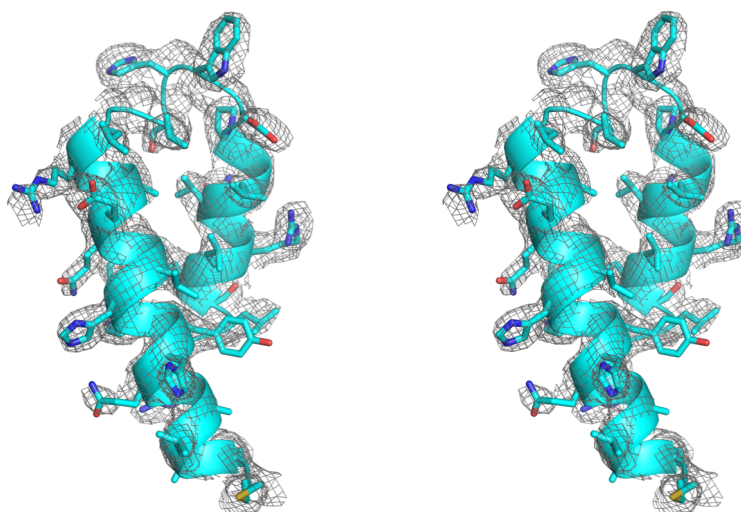
b



c



d



**Supplementary Figure 10. Electron density maps for TssA1<sup>B</sup> Nt1, TssA1<sup>B</sup> CTD, TssA2<sup>A</sup> Nt2 and TssA2<sup>A</sup> CTD.** (a) Electron density visible for residues K72-P115 of helices  $\alpha$ 4- $\alpha$ 5 of TssA1<sup>B</sup> Nt1. (b) Electron density visible for tag residues H301-M302 and residues I303-W347 of helices  $\alpha$ 12- $\alpha$ 13 of TssA1<sup>B</sup> CTD. (c) Electron density visible for residues M284-P315 of helices  $\alpha$ 3- $\alpha$ 4 of the TssA2<sup>A</sup> Nt2 structure. (d) Electron density visible for residues M438-A474 of helices  $\alpha$ 11- $\alpha$ 12 of the TssA2<sup>A</sup> CTD structure. All 2Fo-Fc maps contoured to 1.0  $\sigma$  RMSD.

**Supplementary Table 1. N-terminal amino acid sequence of low mol. wt. His<sub>6</sub>.TssA1<sup>B</sup> degradation products**

| Peptide mol. wt. <sup>a</sup> | N-terminal sequence                              |
|-------------------------------|--------------------------------------------------|
| 23 kDa                        | G-S-S-H-H-H-H- <sup>b</sup><br>H-A-D-X-X-X-      |
| 15 kDa                        | A-A-Q-Q-A-Q-P-E<br>Q-A-Q-P-E-X-I-E-              |
| 12 kDa                        | K-R-D-P-E-H-A-D-D-<br>R-D-P-E-H-A-D-D-           |
| 10 kDa                        | Q-Q-T-A-S-R-P-<br>T-A-S-R-P-P-V-<br>S-R-P-P-V-T- |
| 8 kDa                         | T-I-A-G-I-Q-N-                                   |

<sup>a</sup>Sizes were estimated based on migration in SDS PA gels.

<sup>b</sup>Corresponds to the N-terminus of His<sub>6</sub>.TssA1<sup>B</sup> following removal of the N-terminal methionine.

**Supplementary Table 2. Data collection and refinement statistics for His<sub>6</sub>.TssA1<sup>B</sup> Nt1 (1-255)**

|                                                     | His <sub>6</sub> .TssA1 <sup>B</sup> Nt1 (1-255)<br>Native | His <sub>6</sub> .TssA1 <sup>B</sup> Nt1 (1-255)<br>Peak - Iodine |
|-----------------------------------------------------|------------------------------------------------------------|-------------------------------------------------------------------|
| <b>PDB code</b>                                     | <b>6HS5</b>                                                |                                                                   |
| <b>Data collection<sup>a</sup></b>                  |                                                            |                                                                   |
| Wavelength (Å)                                      | 1.04434                                                    | 1.70000                                                           |
| Space group                                         | P2 <sub>1</sub> 2 <sub>1</sub> 2                           | P2 <sub>1</sub> 2 <sub>1</sub> 2                                  |
| Cell dimensions                                     |                                                            |                                                                   |
| <i>a</i> , <i>b</i> , <i>c</i> (Å)                  | 49.7, 125.7, 45.6                                          | 49.8, 125.5, 45.6                                                 |
| $\alpha$ , $\beta$ , $\gamma$ (°)                   | 90.0, 90.0, 90.0                                           | 90.0, 90.0, 90.0                                                  |
| Resolution range (Å)                                | 49.67-1.80 (1.85-1.80)                                     | 29.64-2.04 (2.09-2.04)                                            |
| <i>R</i> <sub>merge</sub>                           | 0.043 (0.344)                                              | 0.169 (0.865)                                                     |
| <i>I</i> / $\sigma$ <i>I</i>                        | 21.2 (4.4)                                                 | 19.3 (2.8)                                                        |
| Completeness (%)                                    | 99.8 (99.0)                                                | 98.1 (74.2)                                                       |
| Redundancy                                          | 5.4 (5.3)                                                  | 23.0 (15.8)                                                       |
| No. total reflections                               | 146307 (10493)                                             | 427376 (15999)                                                    |
| No. unique reflections                              | 27244 (1966)                                               | 18554 (1011)                                                      |
| <b>Refinement</b>                                   |                                                            |                                                                   |
| Resolution range (Å)                                | 46.2-1.80                                                  |                                                                   |
| No. reflections (Work/Free)                         | 25817/1381                                                 |                                                                   |
| <i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> | 0.177/0.226                                                |                                                                   |
| No. atoms                                           |                                                            |                                                                   |
| Protein                                             | 1991                                                       |                                                                   |
| Ligand/ion <sup>b</sup>                             | 6                                                          |                                                                   |
| Water                                               | 119                                                        |                                                                   |
| <i>B</i> -factors (Å <sup>2</sup> )                 |                                                            |                                                                   |
| Protein                                             | 30.6                                                       |                                                                   |
| Ligand/ion <sup>b</sup>                             | 22.5/20.5                                                  |                                                                   |
| Water                                               | 29.4                                                       |                                                                   |
| R.m.s. deviations                                   |                                                            |                                                                   |
| Bond lengths (Å)                                    | 0.0157                                                     |                                                                   |
| Bond angles (°)                                     | 1.5665                                                     |                                                                   |
| Ramachandran favoured (%) <sup>c</sup>              | 98.79                                                      |                                                                   |
| Ramachandran outliers (%) <sup>c</sup>              | 0.00                                                       |                                                                   |

The number of crystals for the His<sub>6</sub>.TssA1<sup>B</sup> Nt1 (1-255) structure is 1 per data set

<sup>a</sup>Values in parentheses are for highest-resolution shell.

<sup>b</sup>1 x ethylene glycol/2 x calcium ion

<sup>c</sup>Calculated using MolProbity<sup>3</sup>

**Supplementary Table 3. Data collection and refinement statistics for TssA1<sup>B</sup> CTD (303-373)**

|                                                     | TssA1 <sup>B</sup> CTD (303-373)<br>Native | TssA1 <sup>B</sup> CTD (303-373)<br>Peak - Selenomethionine |
|-----------------------------------------------------|--------------------------------------------|-------------------------------------------------------------|
| <b>PDB code</b>                                     | <b>6HS6</b>                                |                                                             |
| <b>Data collection<sup>a</sup></b>                  |                                            |                                                             |
| Wavelength (Å)                                      | 1.70001                                    | 0.97922                                                     |
| Space group                                         | I222                                       | I222                                                        |
| Cell dimensions                                     |                                            |                                                             |
| <i>a</i> , <i>b</i> , <i>c</i> (Å)                  | 46.3, 201.7, 263.7                         | 46.0, 200.3, 263.0                                          |
| $\alpha$ , $\beta$ , $\gamma$ (°)                   | 90.0, 90.0, 90.0                           | 90.0, 90.0, 90.0                                            |
| Resolution range (Å)                                | 41.49-3.08 (3.16-3.08)                     | 100.12-3.03 (3.11-3.03)                                     |
| <i>R</i> <sub>merge</sub>                           | 0.131 (0.786)                              | 0.220 (2.144)                                               |
| <i>I</i> / $\sigma I$                               | 17.5 (3.7)                                 | 9.8 (1.4)                                                   |
| Completeness (%)                                    | 99.9 (100.0)                               | 99.9 (100.0)                                                |
| Redundancy                                          | 12.6 (11.9)                                | 12.9 (13.4)                                                 |
| No. total reflections                               | 297122 (20394)                             | 315386 (23629)                                              |
| No. unique reflections                              | 23602 (1709)                               | 24385 (1759)                                                |
| <b>Refinement</b>                                   |                                            |                                                             |
| Resolution range (Å)                                | 41.49-3.08                                 |                                                             |
| No. reflections (Work/Free)                         | 22387/1215                                 |                                                             |
| <i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> | 0.198/0.242                                |                                                             |
| No. atoms                                           |                                            |                                                             |
| Protein                                             | 4401                                       |                                                             |
| Ligand/ion                                          | 0                                          |                                                             |
| Water                                               | 0                                          |                                                             |
| <i>B</i> -factors (Å <sup>2</sup> )                 |                                            |                                                             |
| Protein                                             | 70.2                                       |                                                             |
| Ligand/ion                                          | -                                          |                                                             |
| Water                                               | -                                          |                                                             |
| R.m.s. deviations                                   |                                            |                                                             |
| Bond lengths (Å)                                    | 0.0114                                     |                                                             |
| Bond angles (°)                                     | 1.6210                                     |                                                             |
| Ramachandran favoured (%) <sup>b</sup>              | 96.05                                      |                                                             |
| Ramachandran outliers (%) <sup>b</sup>              | 0.00                                       |                                                             |

The number of crystals for the TssA1<sup>B</sup> CTD (303-373) structure is 1 per data set

<sup>a</sup>Values in parentheses are for highest-resolution shell.

<sup>b</sup>Calculated using MolProbity<sup>3</sup>

**Supplementary Table 4. Data collection and refinement statistics for His<sub>6</sub>.TssA1<sup>B</sup> CTD H12-H14 (303-358)**

|                                                     | His <sub>6</sub> .TssA1 <sup>B</sup> CTD H12-H14<br>(303-358) |
|-----------------------------------------------------|---------------------------------------------------------------|
| <b>PDB code</b>                                     | <b>6H8E</b>                                                   |
| <b>Data collection<sup>a</sup></b>                  |                                                               |
| Wavelength (Å)                                      | 0.97943                                                       |
| Space group                                         | P6 <sub>2</sub>                                               |
| Cell dimensions                                     |                                                               |
| <i>a</i> , <i>b</i> , <i>c</i> (Å)                  | 65.3, 65.3, 66.1                                              |
| $\alpha$ , $\beta$ , $\gamma$ (°)                   | 90.0, 90.0, 120.0                                             |
| Resolution range (Å)                                | 42.97-2.35 (2.41-2.35)                                        |
| <i>R</i> <sub>merge</sub>                           | 0.094 (0.713)                                                 |
| <i>I</i> / $\sigma I$                               | 17.1 (3.1)                                                    |
| Completeness (%)                                    | 99.9 (99.4)                                                   |
| Redundancy                                          | 8.5 (8.1)                                                     |
| No. total reflections                               | 57299 (4002)                                                  |
| No. unique reflections                              | 6747 (492)                                                    |
| <b>Refinement</b>                                   |                                                               |
| Resolution range (Å)                                | 42.97-2.35                                                    |
| No. reflections (Work/Free)                         | 6372/357                                                      |
| <i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> | 0.193/0.237                                                   |
| No. atoms                                           |                                                               |
| Protein                                             | 897                                                           |
| Ligand/ion                                          | 0                                                             |
| Water                                               | 0                                                             |
| <i>B</i> -factors (Å <sup>2</sup> )                 |                                                               |
| Protein                                             | 45.1                                                          |
| Ligand/ion                                          | -                                                             |
| Water                                               | -                                                             |
| R.m.s. deviations                                   |                                                               |
| Bond lengths (Å)                                    | 0.0129                                                        |
| Bond angles (°)                                     | 1.4320                                                        |
| Ramachandran favoured (%) <sup>b</sup>              | 99.06                                                         |
| Ramachandran outliers (%) <sup>b</sup>              | 0.00                                                          |

The number of crystals for the His<sub>6</sub>.TssA1<sup>B</sup> CTD (303-358) structure is 1

<sup>a</sup>Values in parentheses are for highest-resolution shell.

<sup>b</sup>Calculated using MolProbity<sup>3</sup>

**Supplementary Table 5. Data collection, phasing and refinement statistics for TssA1<sup>B</sup> CTD (303-373) fragment**

|                                                     | TssA1 <sup>B</sup> CTD (303-373) fragment | TssA1 <sup>B</sup> CTD (303-373) fragment - Selenomethionine |                   |                  |
|-----------------------------------------------------|-------------------------------------------|--------------------------------------------------------------|-------------------|------------------|
| <b>PDB code</b>                                     | <b>6H8F</b>                               |                                                              |                   |                  |
| <b>Data collection<sup>a</sup></b>                  |                                           |                                                              |                   |                  |
|                                                     |                                           | <i>Peak</i>                                                  | <i>Inflection</i> | <i>Remote</i>    |
| Wavelength (Å)                                      | 0.92000                                   | 0.97934                                                      | 0.97949           | 0.96411          |
| Space group                                         | I222                                      | I222                                                         | I222              | I222             |
| Cell dimensions                                     |                                           |                                                              |                   |                  |
| <i>a</i> , <i>b</i> , <i>c</i> (Å)                  | 48.3, 62.7, 65.7                          | 47.8, 62.6, 65.6                                             | 47.8, 62.8, 65.8  | 47.7, 62.9, 65.7 |
| $\alpha$ , $\beta$ , $\gamma$ (°)                   | 90.0, 90.0, 90.0                          | 90.0, 90.0, 90.0                                             | 90.0, 90.0, 90.0  | 90.0, 90.0, 90.0 |
| Resolution range (Å)                                | 38.90-1.78                                | 16.79-1.71                                                   | 38.64-1.87        | 38.61-2.04       |
|                                                     | (1.83-1.78)                               | (1.75-1.71)                                                  | (1.92-1.87)       | (2.09-2.04)      |
| <i>R</i> <sub>merge</sub>                           | 0.068 (0.697)                             | 0.059 (0.512)                                                | 0.065 (0.739)     | 0.072 (0.703)    |
| <i>I</i> / $\sigma$ <i>I</i>                        | 13.2 (2.0)                                | 21.4 (2.7)                                                   | 22.0 (2.9)        | 21.7 (4.0)       |
| Completeness (%)                                    | 97.0 (99.3)                               | 97.9 (83.9)                                                  | 100.0 (100.0)     | 100.0 (100.0)    |
| Redundancy                                          | 4.5 (4.6)                                 | 11.2 (5.6)                                                   | 12.5 (9.6)        | 12.7 (13.1)      |
| No. total reflections                               | 42707 (3238)                              | 120101 (3596)                                                | 105843 (5954)     | 83404 (6305)     |
| No. unique reflections                              | 9502 (700)                                | 10692 (644)                                                  | 8487 (618)        | 6577 (482)       |
| <b>Refinement</b>                                   |                                           |                                                              |                   |                  |
| Resolution (Å)                                      | 38.90-1.78                                |                                                              |                   |                  |
| No. reflections (Work/Free)                         | 9048/454                                  |                                                              |                   |                  |
| <i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> | 0.189/0.237                               |                                                              |                   |                  |
| No. atoms                                           |                                           |                                                              |                   |                  |
| Protein                                             | 752                                       |                                                              |                   |                  |
| Ligand/ion                                          | 0                                         |                                                              |                   |                  |
| Water                                               | 39                                        |                                                              |                   |                  |
| <i>B</i> -factors (Å <sup>2</sup> )                 |                                           |                                                              |                   |                  |
| Protein                                             | 27.6                                      |                                                              |                   |                  |
| Ligand/ion                                          | -                                         |                                                              |                   |                  |
| Water                                               | 27.6                                      |                                                              |                   |                  |
| R.m.s deviations                                    |                                           |                                                              |                   |                  |
| Bond lengths (Å)                                    | 0.0117                                    |                                                              |                   |                  |
| Bond angles (°)                                     | 1.3164                                    |                                                              |                   |                  |
| Ramachandran favoured (%) <sup>b</sup>              | 100.00                                    |                                                              |                   |                  |
| Ramachandran outliers (%) <sup>b</sup>              | 0.00                                      |                                                              |                   |                  |

The number of crystals for the TssA1<sup>B</sup> CTD (303-373) fragment structure is 1 per data set

<sup>a</sup>Values in parentheses are for highest-resolution shell.

<sup>b</sup>Calculated using MolProbity<sup>3</sup>

**Supplementary Table 6. Data collection and refinement statistics for TssA2<sup>A</sup> Nt1-Nt2.His<sub>6</sub> (1-374) and TssA2<sup>A</sup> His<sub>6</sub>.Nt2 (231-374)**

|                                                     | TssA2 <sup>A</sup> Nt1-Nt2.His <sub>6</sub> (1-374) | TssA2 <sup>A</sup> His <sub>6</sub> .Nt2 (231-374) -<br>Mercury |
|-----------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------------------|
| <b>PDB code</b>                                     | <b>6G7B</b>                                         |                                                                 |
| <b>Data collection<sup>a</sup></b>                  |                                                     |                                                                 |
| Wavelength (Å)                                      | 0.9795                                              | 1.0088                                                          |
| Space group                                         | P2 <sub>1</sub>                                     | P2 <sub>1</sub>                                                 |
| Cell dimensions                                     |                                                     |                                                                 |
| <i>a</i> , <i>b</i> , <i>c</i> (Å)                  | 39.8, 101.3, 78.4                                   | 45.8, 40.0, 100.5                                               |
| $\alpha$ , $\beta$ , $\gamma$ (°)                   | 90.0, 104.6, 90.0                                   | 90.0, 102.5, 90.0                                               |
| Resolution range (Å)                                | 42.13-1.76 (1.79-1.76)                              | 44.71-2.16 (2.23-2.16)                                          |
| <i>R</i> <sub>merge</sub>                           | 0.065 (0.611)                                       | 0.162 (1.492)                                                   |
| <i>I</i> / $\sigma I$                               | 7 (1.2)                                             | 9.4 (1.2)                                                       |
| Completeness (%)                                    | 99.9 (98.7)                                         | 95.9 (71.9)                                                     |
| Redundancy                                          | 3.6 (3.3)                                           | 12.4 (8.6)                                                      |
| No. total reflections                               | 214716 (9711)                                       | 231080 (8786)                                                   |
| No. unique reflections                              | 59629 (2971)                                        | 18671 (1026)                                                    |
| <b>Refinement</b>                                   |                                                     |                                                                 |
| Resolution range (Å)                                | 101.32-1.76                                         |                                                                 |
| No. reflections (Work/Free)                         | 56717/2805                                          |                                                                 |
| <i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> | 0.272/0.306                                         |                                                                 |
| No. atoms                                           |                                                     |                                                                 |
| Protein                                             | 4584                                                |                                                                 |
| Ligand/ion                                          | 0                                                   |                                                                 |
| Water                                               | 380                                                 |                                                                 |
| <i>B</i> -factors (Å <sup>2</sup> )                 |                                                     |                                                                 |
| Protein                                             | 22.9                                                |                                                                 |
| Ligand/ion                                          | -                                                   |                                                                 |
| Water                                               | 29.3                                                |                                                                 |
| R.m.s. deviations                                   |                                                     |                                                                 |
| Bond lengths (Å)                                    | 0.0175                                              |                                                                 |
| Bond angles (°)                                     | 1.702                                               |                                                                 |
| Ramachandran favoured (%) <sup>b</sup>              | 98.8                                                |                                                                 |
| Ramachandran outliers (%) <sup>b</sup>              | 0.00                                                |                                                                 |

The number of crystals for the TssA2<sup>A</sup> Nt1-Nt2.His<sub>6</sub> (1-374) structure is 1 per data set

<sup>a</sup>Values in parentheses are for highest-resolution shell.

<sup>b</sup>Calculated using MolProbity<sup>3</sup>

**Supplementary Table 7. Data collection and refinement statistics for TssA2<sup>A</sup> His<sub>6</sub>.Nt2-CTD (223-478)**

|                                                     | TssA2 <sup>A</sup> His <sub>6</sub> .Nt2-CTD (223-478) |
|-----------------------------------------------------|--------------------------------------------------------|
| <b>PDB code</b>                                     | <b>6G7C</b>                                            |
| <b>Data collection<sup>a</sup></b>                  |                                                        |
| Wavelength (Å)                                      | 0.97623                                                |
| Space group                                         | P2 <sub>1</sub>                                        |
| Cell dimensions                                     |                                                        |
| <i>a</i> , <i>b</i> , <i>c</i> (Å)                  | 70.0, 202.1, 139.0                                     |
| $\alpha$ , $\beta$ , $\gamma$ (°)                   | 90.0, 90.7, 90.0                                       |
| Resolution range (Å)                                | 139.00-3.13 (3.21-3.13)                                |
| <i>R</i> <sub>merge</sub>                           | 0.113 (1.368)                                          |
| <i>I</i> / $\sigma I$                               | 5.8 (0.9)                                              |
| Completeness (%)                                    | 99.9 (100.0)                                           |
| Redundancy                                          | 3.7 (3.6)                                              |
| No. total reflections                               | 253133 (18358)                                         |
| No. unique reflections                              | 67977 (5030)                                           |
| <b>Refinement</b>                                   |                                                        |
| Resolution range (Å)                                | 202.08-3.13                                            |
| No. reflections (Work/Free)                         | 64474/3380                                             |
| <i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> | 0.255/0.328                                            |
| No. atoms                                           |                                                        |
| Protein                                             | 18363                                                  |
| Ligand/ion                                          | 0                                                      |
| Water                                               | 0                                                      |
| <i>B</i> -factors (Å <sup>2</sup> )                 |                                                        |
| Protein                                             | 99.3                                                   |
| Ligand/ion                                          | -                                                      |
| Water                                               | -                                                      |
| R.m.s. deviations                                   |                                                        |
| Bond lengths (Å)                                    | 0.011                                                  |
| Bond angles (°)                                     | 1.60                                                   |
| Ramachandran favoured (%) <sup>b</sup>              | 90.5                                                   |
| Ramachandran outliers (%) <sup>b</sup>              | 1.35                                                   |

The number of crystals for the TssA2<sup>A</sup> His<sub>6</sub>.Nt2-CTD (223-478) structure is 1

<sup>a</sup>Values in parentheses are for highest-resolution shell.

<sup>b</sup>Calculated using MolProbity<sup>3</sup>

**Supplementary Table 8. Bacterial strains**

| Strain                | Genotype/Description <sup>a</sup>                                                                                                                                            | Source or reference |
|-----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| <i>B. cenocepacia</i> |                                                                                                                                                                              |                     |
| H111                  | CF isolate, prototroph                                                                                                                                                       | (4)                 |
| H111ΔtssM             | H111 containing an in-frame deletion within <i>tssM</i>                                                                                                                      | This study          |
| H111-tssA::Tp         | H111 containing a Tp <sup>R</sup> cassette insertion within <i>tssA</i>                                                                                                      | This study          |
| <i>A. hydrophila</i>  |                                                                                                                                                                              |                     |
| ATCC 7966             | Wild type. Prototroph.                                                                                                                                                       | (5)                 |
| <i>E. coli</i>        |                                                                                                                                                                              |                     |
| JM83                  | F <sup>-</sup> <i>ara</i> Δ( <i>lac-proAB</i> ) <i>rpsL</i> φ80d <i>lacZ</i> Δ <i>M15</i> (Sm <sup>R</sup> )                                                                 | (6)                 |
|                       | F <sup>-</sup> <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> )                                                             |                     |
| DH5α                  | <i>supE44 relA1</i> Δ( <i>lacZYA-argF</i> )U169 <i>deoR phoA</i> λ <sup>-</sup> φ80d <i>lacZ</i> Δ <i>M15</i>                                                                | (7)                 |
| S17-1(λpir)           | <i>thi proA hsdR recA</i> RP4-2- <i>tet</i> ::Mu-1 <i>kan</i> ::Tn7 integrant λpir (Tp <sup>R</sup> Sm <sup>R</sup> )                                                        | (8)                 |
| SM10(λpir)            | <i>thi-1 thr leu tonA lacY supE recA</i> RP4-2-Tc::Mu λpir (Km <sup>R</sup> )                                                                                                | (8)                 |
| BTH101                | F <sup>-</sup> , <i>cya-99 araD139 galE15 galK16 rpsL1</i> (Sm <sup>R</sup> ) <i>hsdR2 mcrA1 mcrB1</i>                                                                       | (9)                 |
| BL21(DE3)             | F <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>+</sup> ) <i>dcm gal</i> λDE3                                                   | (10)                |
| ER2523                | <i>fhuA2 [lon] ompT gal sulA11 (mcr-73::mini-Tn10)2</i> (Tc <sup>S</sup> ) [ <i>dcm</i> ] <i>zgb-210::Tn10</i> (Tc <sup>S</sup> ) <i>endA1</i> Δ( <i>mcrC-mrr</i> )114::IS10 | New England Biolabs |

<sup>a</sup>Km<sup>R</sup>, kanamycin resistant; Rf<sup>R</sup>, rifampicin resistant; Sm<sup>R</sup>, streptomycin resistant; Tc<sup>S</sup>, tetracycline sensitive; Tp<sup>R</sup>, trimethoprim resistant.

**Supplementary Table 9. Oligonucleotides**

| Oligo/Primer name                                      | Sequence (5'-3') <sup>a,b,c</sup>                                                                |
|--------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| <b>Vector modification</b>                             |                                                                                                  |
| delacO1for                                             | GATCACGGGATCTCGACGCTCTCCCTATTAGGAAATTAATACG                                                      |
| delacO1for2                                            | ACTCACTATAGGGACCTGTAGAAATAATTTTGTTTAACTTTAATA<br>AGGAGATATAC                                     |
| delacO1rev                                             | CATGGTATATCTCCTTATTAAGTTAAACAAAATTATTTCTACAG<br>GTCC                                             |
| delacO1rev2                                            | CTATAGTGAGTCGTATTAATTTCTAATAGGGAGAGCGTCGAGA<br>TCCCGT                                            |
| delacO2for                                             | GTACACGGCCGCATAATCGAAATTAATACGACT                                                                |
| delacO2for2                                            | CACTATAGGGACCATCTTAGTATATTAGTTAAGTATAAGAAGGA<br>GATATACA                                         |
| delacO2rev                                             | TATGTATATCTCCTTCTTATACTTAACTAATAT                                                                |
| delacO2rev2                                            | ACTAAGATGGTCCCTATAGTGAGTCGTATTAATTTTCGATTATGCG<br>GCCGT                                          |
| pMALHisFor                                             | GCGC <u>CAATTG</u> ACCAACAAGGACCATAGATTATGAAAATCCATCACCAT<br>CACCATCACGAAGAAGGTAAACTGG<br>TAATCT |
| pMALHisRev                                             | TGGCGGGTTCGGCAGCAGATCT                                                                           |
| <b>Construction of <i>tssA1<sup>B</sup></i> mutant</b> |                                                                                                  |
| iotAfor                                                | GCGC <u>AAGCTT</u> CACGCGACATCTCATGCATC                                                          |
| iotAfor3                                               | GCGC <u>AAGCTTCCGCGG</u> AAATTTCGACGCGATCCAGGAC                                                  |
| iotArev                                                | GCGC <u>GGATCCCC</u> CGTCTTGCGT <u>TTA</u> CGACT                                                 |
| iotArev2                                               | ATCACGAAGAGCATTCCGCC                                                                             |
| <b>Construction of <i>tssM</i> mutant</b>              |                                                                                                  |
| tssMfor                                                | GCGCTCTAGAGGAACCTGAACGTCCTATGC                                                                   |
| tssMrev2                                               | GCGCGGTACCTCATTGCGCCCTCTGTGCAT                                                                   |
| tssM-OPfor                                             | TCATCCCGTTTGACAGCATG                                                                             |
| tssM-OPrev2                                            | ATCTTGCCGAAGTAGGCGATTT                                                                           |
| <b>Subunit expression for purification</b>             |                                                                                                  |
| TssA1 <sup>B</sup>                                     |                                                                                                  |

|                                         |                                                                                 |
|-----------------------------------------|---------------------------------------------------------------------------------|
| tssAnonHisFor                           | GCGCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGC<br>CGATCAATCTCCCCGA           |
| pET14b-iotAfor                          | GCGCCATATGCCGATCAATCTCCCCGA                                                     |
| pET14b-iotArev                          | GCGCGGATCCTGCGTTTACGACTGCTCGTC                                                  |
| TssAlinkerHis1for                       | GCGCATCACCATCACCATCACGCGCAGCAGGCCAGCCCGA                                        |
| TssAlinkerHis1rev                       | CGCGTGATGGTGATGGTGATGCGCCTGCGTGTGCGGCGCGC                                       |
| pET14b-ACTDfor                          | GCGCCATATGCCTCCGGTGACGCAGACGAT                                                  |
| pACYC-tssA.CTDfor                       | GCGCCATATGAGCAGCCATCATCATCATCACAGCATCCAGAACC<br>GTGCGCAGGC                      |
| pACYC-<br>tssA.CTDH1rev2                | GCGCGGATCCTTAGACCGGGCTGTGCGGCTCGG                                               |
| pACYC-<br>tssA.CTDH2rev2                | GCGCGGATCCTTACCCTTGTGCAGCGGCATGT                                                |
| pACYC-<br>tssA.CTDH3rev2                | GCGCGGATCCTTACAGCGAGCCGTCGTCCTTCA                                               |
| TssA2 <sup>A</sup>                      |                                                                                 |
| pET21a-<br>AHA1844.Nt1Nt2.for           | ATATATACATATGAGCTATCAACACCCCTGGTGTGCAC                                          |
| pET21a-<br>AHA1844.Nt1Nt2.rev           | TATATTATATACTCGAGCAGCCAGCGGCTGCA                                                |
| pACYC-<br>AHA1844.CTDSD2for             | GCGCGGATCCGAGTGCGGGCATTGGCGAGGC                                                 |
| pACYC-AHA1844.rev                       | GCGCAAGCTTTCAATTCGACAACGGCGCCG                                                  |
| TssD                                    |                                                                                 |
| pET-His-iotDfor2                        | TATGCGAAGGGCCATGTTACAT                                                          |
| pET14b-iotDrev                          | GCGCGGATCCTTAGACCGCGTAGGTCTT                                                    |
| <b>Subunit expression<br/>for co-IP</b> |                                                                                 |
| TssA.NdeI.FLAG.for                      | GCGCCATATGGATTATAAAGACGACGATGATAAACCGATCAATCTCCC<br>CGAGCT                      |
| TssA.BglII.rev                          | GCGCAGATCTTGCGTTTACGACTGCTCGTC                                                  |
| tssC.NcoI.for                           | GCGCCCATGGCAAACCAGCAAACGGCTGC                                                   |
| tssC.VSVg.BamHI.rev                     | GCGCGGATCCTTACTTACCCAGGCGGTTCAATTCGATATCAGTGTATG<br>AATTTGCCGGTTTGG             |
| tssD.forpACYC.Nterm<br>VSVgTag          | GCGCCCATGGCATACACTGATATCGAAATGAACCGCCTGGGTAAGAT<br>GTTACATATGCACTTGCAGTTTGGTAGT |
| tssD. BamHI.rev                         | GCGCGGATCCTTAGACCGCGTAGGTCTTGT                                                  |

|                             |                                                                        |
|-----------------------------|------------------------------------------------------------------------|
| pMALtssEfor                 | GCGCCATATGAAACGATTCTGAACCCAG                                           |
| pMALtssErev                 | GCGCGGATCCCGGCTTGATTACTGCATGC                                          |
| tssF.NcoI.for               | GCGCCCATGGAAGAATTGCTGCCGTATTA                                          |
| tssF.HA.HindIII.rev         | GCGCAAGCTTCTAAGCGTAGTCTGGGACGTCGTATGGGTACGCCAG<br>GATCGATTTCGC         |
| tssF.NdeI.HA.for            | GCGCCATATGTACCCATACGACGTCCCAGACTACGCTGAAGAATTGC<br>TGCCGTATTA          |
| C-term-iotFrev              | GCGCGGTACCATCGACTACGCCAGGATCGA                                         |
| tssl.BspHI.VSVgtag.<br>For  | GCGCTCATGAGCTTACACTGATATCGAAATGAACCGCCTGGGTAAGAT<br>GGATGCGCACAGCATGAT |
| BCAM0148gp5.BglII.<br>rev   | GCGCAGATCTTTAAACTCCTGGGACCGGTAGC                                       |
| TssKforpET.Nterm<br>VSVgTag | GCGCCCATGGCATACACTGATATCGAAATGAACCGCCTGGGTAAGAT<br>GAGTTATTCGGCCAAGGT  |
| TssK.BglII.rev              | GCGCAGATCTTCATGATGTGACCGCGATCA                                         |
| TssLforpET.Nterm<br>VSVgTag | GCGCCCATGGCATACACTGATATCGAAATGAACCGCCTGGGTAAGAT<br>GAGCTACGCGCCTTCCTT  |
| TssL.Rev                    | GCGCAGATCTTTACTTCAAGCGGTGCGCGAT                                        |
| TssM1.VSVg.NcoI.for         | GCGCCCATGGCATACACTGATATCGAAATGAACCGCCTGGGTAAGGT<br>GAAGCGTGCGAACCAGCA  |
| TssM1.HindIII.rev           | GCGCAAGCTTTTGAAGGTCGCGTAGCGCAGCC                                       |
| <b>Two-hybrid system</b>    |                                                                        |
| TssA1 <sup>B</sup>          |                                                                        |
| Cterm-iotAfor               | GCGCCTGCAGGGATGCCGATCAATCTCCCCGA                                       |
| Cterm-iotArev               | GCGCTCTAGATGCGTTTACGACTGCTCGTC                                         |
| Nterm-iotAfor               | GCGCAAGCTTGATGCCGATCAATCTCCCCGA                                        |
| N-iotAfullrev               | GCGCGGATCCGACGACTGCTCGTCGGGCCG                                         |
| C-iotAlongNTDrev            | GCGCTCTAGAGGTTAGCTGCCGGTATAGCCCTGTT                                    |
| N-iotAlongNTDrev            | GCGCGGATCCTCGCTGCCGGTATAGCCCTGTT                                       |
| C-term-iotAfor              | GCGCCTGCAGGGATGCCGATCAATCTCCCCGA                                       |
| C-iotAshortCTDfor           | GCGCCTGCAGGTCCTCCGGTGACGCAGACGAT                                       |
| N-iotAshortCTDfor           | GCGCAAGCTTTCCTCCGGTGACGCAGACGAT                                        |
| TssA2 <sup>A</sup>          |                                                                        |
| AHA1844fullfor              | GCGCTCTAGAGATGAGCTATCAACACCCCT                                         |
| AHA1844-Crev2               | GCGCGGTACCGGCATTCATTTTCGACAACGG                                        |
| AHA1844-Nrev                | GCGCGGTACCCATTTTCGACAACGGCGCCGC                                        |

|                     |                                               |
|---------------------|-----------------------------------------------|
| AHA1844NTDrev       | GCGCGGTACC <u>TTA</u> GCTGGAAGTGTCTGACGTCGACG |
| AHA1844NTDrev2      | GCGCGGTACC <u>CGT</u> GGAAGTGTCTGACGTCGACG    |
| AHA1844for          | GCGCTCTAGACGTCGACGTCGACAGTTCCA                |
| AHA1844CTDrev       | GCGCGGTACC <u>TTA</u> GGCCTCGCCAATGCCC GCACT  |
| AHA1844for2         | GCGCTCTAGAAAGTGCGGGCATTGGCGAGGC               |
| Other T6SS subunits |                                               |
| Cterm-iotBfor       | GCGCTCTAGACGTCGAGATGGCCAAGAAAGA               |
| Cterm-iotBrev       | GCGCGAATTCTTCG <u>TCA</u> TTCGCCGTCTTTGT      |
| Nterm-iotBfor       | GCGCGCATGCCACCGCAACTGACGCGATAAC               |
| N-iotBfullrev       | GCGCTCTAGAGATTGCGCCGTCTTTGTCTG                |
| Cterm-iotCfor       | GCGCCTGCAGAGATGAACCAGCAAACGGCT                |
| Cterm-iotCrev       | GCGCTCTAGACTCGC <u>TTA</u> TGAATTTGCCGG       |
| Nterm-iotCfor       | GCGCAAGCTTGCAGATGAACCAGCAAACGGCT              |
| Nterm-tssCrev.new   | GCGCGGATCCTTGAATTTGCCGGTTTGGGCA               |
| Cterm-tssCfor.new   | GCGCCTGCAGAATGAACCAGCAAACGGCT                 |
| pUT18C-iotDfor      | GCGCCTGCAGGAGTCGAAGGGCCATGTTACA               |
| Cterm-iotDrev       | GCGCTCTAGAGT <u>TTA</u> GACCGCGTAGGTCTTGT     |
| Nterm-iotDfor       | GCGCAAGCTTGCAGTTGCAGTTTGGTAGTCC               |
| Nterm-iotDrev       | GCGCGGATCCGCGTAGGTCTTGTCTGTTCTT               |
| Cterm-iotEfor       | GCGCTCTAGAGGCGATGAAACGATTCTGAACCC             |
| Cterm-iotErev       | GCGCGAATTCTCGGCTTGAT <u>TTA</u> CTGCATGC      |
| Nterm-iotEfor       | GCGCAAGCTTGACGATGAAACGATTCTGAACCC             |
| N-iotEfullrev       | GCGCGGATCCGACTGCATGCGTGC CGC GCG T            |
| Cterm-iotFfor       | GCGCTCTAGAGACGATGGAAGAATTGCTGCCG              |
| Cterm-iotFrev       | GCGCGGTACCATCGA <u>CTA</u> CGCCAGGATCGAT      |
| Nterm-iotFfor       | GCGCAAGCTTGCAGATGGAAGAATTGCTGCCGT             |
| N-iotFfullrev       | GCGCTCTAGAGACGCCAGGATCGATTGCC                 |
| C-iotGfrag1for      | GCGCCTCTAGAGATGCAAGCCCCGAACCGGCG              |
| Cterm-iotGrev       | GCGCGAATTCTTGGGTCT <u>TCA</u> GTGAATCACGTG    |
| N-iotGfrag1for      | GCGCAAGCTTGATGCAAGCCCCGAACCGGCG               |
| Nterm-iotGrev2      | GCGCGGATCCTCGTGAATCACGTGCAGTTCGTA             |
| Tsslfor             | GCGCTCTAGAGATGGATGCGCACAGCATGAT               |
| C-tsslrev           | GCGCCCCGGGAGATCTAGT <u>CTA</u> TGCTTCCGACGAAG |
| N-tsslrev           | GCGCCCCGGGAAAGATCTCCTGCTTCCGACGAAGGACTGA      |
| Rev.gp5             | GCGCAGATCT <u>TTA</u> AAACTCCTGGGACCGGTAG     |
| For1tssj            | GCGCCTGCAGAACTTCTGGCCGGATGCGCGGC              |

|            |                                             |
|------------|---------------------------------------------|
| C-tssJrev  | GCGCGGATCC <b>TTA</b> ACCGCAGGAGACCGAAG     |
| For2tssj   | GCGCCTGCAGACTTCTGGCCGGATGCGCGGC             |
| N-tssJrev  | GCGCGGATCCAAACCGCAGGAGACCGAAGACA            |
| tssKfor    | GCGCGGATCCAATGAGTTATTCGGCCAAGGT             |
| C-tssKrev  | GCGCGGTACCT <b>TCAT</b> GATGTGACCGCGATCA    |
| N-tssKrev  | <u>GGTACCAATGATGTGACCGCGATCAGTT</u>         |
| tssLfor    | CGCGTCTAGAAATGAGCTACGCGCCTTCCTT             |
| C-tssLrev  | GCGCGGTACCT <b>TTA</b> CTTCAAGCGGTGCGCGATCT |
| N-tssLrev  | GCGCGGTACCACTTCAAGCGGTGCGCGATCT             |
| tssM1for   | CGCTCTAGAAGTGAAGCGTGCGAACCAGCA              |
| C-tssM1rev | CGCGGTACCT <b>TTA</b> GAAGAAGGTCGCGTAGCGCA  |
| N-tssM1rev | CGCGGTACCAAAAGAAGGTCGCGTAGCGCAG             |
| tssM2for   | CGCGGATCCCACGATCGGCAACCAGCAGCT              |
| C-tssM2rev | GCGGGTACCGT <b>TCAT</b> TGCGCCCTCTGTGCA     |
| N-tssM2rev | GCGGGTACCAATTGCGCCCTCTGTGCATTG              |

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<sup>a</sup>Sequences specifying restriction endonuclease cleavage sites are underlined.

<sup>b</sup>Sequences specifying affinity or epitope tags are shown in italic font.

<sup>c</sup>Stop codons are shown in red font.

**Supplementary Table 10. Plasmids**

| Plasmid                           | Description <sup>a</sup>                                                                                                                                                                                                                          | Source or reference |
|-----------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| pBBR1MCS                          | Mobilisable BHR cloning vector (Cm <sup>R</sup> )                                                                                                                                                                                                 | (11)                |
| pBBR1MCS-TssA1 <sup>B</sup>       | Complementation plasmid. Constructed by amplifying <i>B. cenocepacia</i> <i>tssA</i> with primers <i>iotAfor</i> and <i>iotArev</i> , restricting with <i>HindIII</i> and <i>BamHI</i> , and ligating the amplicon to the same sites of pBBR1MCS. | This study          |
| pBBR1MCS-‘tssA1 <sup>B</sup>      | The final 347 codons of <i>B. cenocepacia</i> <i>tssA</i> were amplified with primers <i>iotAfor3</i> and <i>iotArev</i> , the amplicon was cut with <i>HindIII</i> and <i>BamHI</i> , and ligated between the same sites of pBBR1MCS.            | This study          |
| p34E-Tp                           | <i>dfrB2</i> (Tp <sup>R</sup> ) cassette vector (Ap <sup>R</sup> , Tp <sup>R</sup> )                                                                                                                                                              | (12)                |
| pBBR1MCS-‘tssA1 <sup>B</sup> ::Tp | pBBR1MCS-‘tssA3 with Tp <sup>R</sup> cassette from p34E-Tp inserted into the <i>EcoRI</i> site within the <i>tssA</i> coding sequence.                                                                                                            | This study          |
| pSHAFT2                           | R6K-derived suicide vector for allelic replacement (Ap <sup>R</sup> , Cm <sup>R</sup> )                                                                                                                                                           | (13)                |
| pSHAFT2-‘tssA1 <sup>B</sup> ::Tp  | Disrupted <i>tssA1<sup>B</sup></i> allele was removed from pBBR1MCS-‘tssA1 <sup>B</sup> ::Tp with <i>XhoI</i> and <i>NotI</i> , and ligated to the <i>Sall</i> and <i>NotI</i> sites of pSHAFT2.                                                  | This study          |
| p34E-TpTer                        | p34E containing <i>dfrB2</i> gene fused to <i>rrnB</i> T1T2 terminators (Ap <sup>R</sup> , Tp <sup>R</sup> )                                                                                                                                      | (13)                |
| pEX18Tp-pheS                      | Gene replacement vector with ColE1-derived replicon, and mutated phenylalanyl tRNA synthase $\alpha$ -subunit gene, <i>pheS*</i> , for cPhe counter- selection (Tp <sup>R</sup> )                                                                 | (14)                |
| pEX18Tp- $\Delta$ rrnB-pheS       | pEX18Tp-pheS derivative with <i>AfeI</i> - <i>BsaI</i> restriction fragment removed (Tp <sup>R</sup> )                                                                                                                                            | This study          |
| pSNUFF                            | pEX18Tp- $\Delta$ rrnB-pheS derivative containing <i>dfrB2</i> - <i>rrnB</i> T1T2 cassette in replacement of <i>SpeI</i> - <i>EcoRV</i> restriction fragment (Tp <sup>R</sup> )                                                                   | This study          |
| pBBR1-tssM(-)                     | pBBR1MCS containing <i>tssM</i> amplified as a 3,943 bp DNA fragment with primers <i>tssMfor</i> and                                                                                                                                              | This study          |

|                           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                     |
|---------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
|                           | tssMrev2, and ligated between the <i>Xba</i> I and <i>Kpn</i> I sites                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                     |
| pBBR1- $\Delta$ tssM      | pBBR1-tssM(-) with a 2133 bp in-frame deletion introduced into the <i>tssM</i> ORF by restriction with <i>Xho</i> I followed by religation                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | This study          |
| pSNUFF- $\Delta$ tssM     | pSNUFF containing $\Delta$ tssM allele from pBBR1- $\Delta$ tssM ligated between the <i>Xba</i> I and <i>Kpn</i> I sites                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | This study          |
| <b>Protein expression</b> |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                     |
| pET14b                    | T7 promoter expression vector allowing for incorporation of N-terminal His <sub>6</sub> tag, pMB1 origin (Ap <sup>R</sup> )                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | Novagen             |
| pET-21a                   | T7 promoter expression plasmid for incorporation of C-terminal His <sub>6</sub> tag, pMB1 origin (Ap <sup>R</sup> )                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Novagen             |
| pACYCDuet-1               | Dual T7-lac promoter expression vector, <i>lacI</i> , p15A origin (Cm <sup>R</sup> )                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | Novagen             |
| pETDuet-1                 | Dual T7-lac promoter expression vector, <i>lacI</i> , pMB1 origin (Ap <sup>R</sup> )                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | Novagen             |
| pETDuet $\Delta$ O        | pETDuet-1 lacking the <i>lac</i> operators at the two T7 promoters and containing a partial deletion of the <i>lacI</i> gene. Deletion of <i>lacO</i> at first T7 promoter and part of the <i>lacI</i> gene involved replacement of the 572 bp region between the <i>Bcl</i> I- <i>Not</i> I sites by annealed oligonucleotides delacO1for, delacO1for2, delacO1rev and delacO1rev2. Deletion of the <i>lacO</i> at the downstream T7 promoter was carried out by replacing the 101 bp region between the <i>Bsr</i> GI- <i>Nde</i> I sites by annealed oligonucleotides delacO2for, delacO2for2, delacO2rev and delacO2rev2. For both manipulations, the 'for2' and 'rev2' primers were kinased to facilitate ligation of the assembled double-stranded oligonucleotide to the vector. | This study          |
| pMAL-c5X                  | Plasmid for generating fusions of MalE (MBP) to the N-terminus of target proteins. <i>malE</i> is under P <sub>tac</sub> control. Contains <i>lacI</i> under P <sub>lacIQ</sub> control (Ap <sup>R</sup> )                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | New England Biolabs |

|                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |            |
|---------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| pMAL-c5X-His <sub>6</sub>                   | pMAL-c5X encoding MBP with a hexa-histidine tag located between amino acids 3 and 4. Constructed by replacing the 390 bp <i>MfeI</i> - <i>BglII</i> fragment (that includes the <i>malE</i> Shine-Dalgarno sequence and first ~360 bp of <i>malE</i> ) with a <i>MfeI</i> - <i>BglII</i> DNA fragment generated by PCR using pMAL-c5X as template and primers pMALHisFor and pMALHisRev.                                                                                                                     | This study |
| pET14b-TssA1 <sup>B</sup>                   | pET14b encoding native TssA1 <sup>B</sup> . <i>B. cenocepacia</i> <i>tssA</i> was amplified with primers tssAnonHisFor and pET14b-iotArev, cut with <i>XbaI</i> and <i>Bam</i> HI, and ligated between the same sites of pET14b                                                                                                                                                                                                                                                                              | This study |
| pET14b-His <sub>6</sub> .TssA1 <sup>B</sup> | pET14b encoding N-terminal His-tagged TssA1 <sup>B</sup> . <i>B. cenocepacia</i> <i>tssA</i> was amplified with primers pET14b-iotAfor and pET14b-iotArev, cut with <i>NdeI</i> and <i>Bam</i> HI, and ligated between the same sites of pET14b. The encoded product contains a 20 amino acid N-terminal tag that harbours a hexahistidine tag and a thrombin cleavage site.                                                                                                                                 | This study |
| pACYCDuet-linkerHis1.TssA1 <sup>B</sup>     | pACYCDuet-1 encoding <i>B. cenocepacia</i> TssA with 6 consecutive histidine codons located between codons 262-263. Constructed by SOE PCR using two pairs of primers (pET14b-iotAfor and TssAlinkerHis1rev, and TssAlinkerHis1for and pET14b-iotArev) in which the products from the first pair of PCRs were combined and spliced in a second PCR using pET14b-iotAfor and pET14b-iotArev. The resulting amplicon was cut with <i>NdeI</i> and <i>BglII</i> , and ligated to the same sites of pACYCDuet-1. | This study |
| pMAL-c5X-TssA1 <sup>B</sup> .CTD            | pMAL-c5X encoding a MBP-TssA1 <sup>B</sup> CTD fusion. DNA encoding amino acids 294-373 of <i>B. cenocepacia</i> <i>tssA</i> was amplified with primers pET14b-ACTDfor and pET14b-iotArev, the product was restricted with <i>NdeI</i> and <i>Bam</i> HI, and ligated between the same sites of pMAL-c5X.                                                                                                                                                                                                    | This study |

|                                                            |                                                                                                                                                                                                                                                                                                                                                            |            |
|------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
|                                                            | Following cleavage of the MBP fusion with Factor Xa, the released TssA <sub>CTD</sub> contains four additional non-native amino acids (ISHM) at the N-terminus.                                                                                                                                                                                            |            |
| pMAL-c5X-TssA1 <sup>B</sup> .CTD3                          | pMAL-c5X encoding MBP fused to amino acids 303-373 of <i>B. cenocepacia</i> TssA1 <sup>B</sup> .                                                                                                                                                                                                                                                           | (2)        |
| pMAL-c5X-His <sub>6</sub> .TssA1 <sup>B</sup> .CTD H12     | pMAL-c5X encoding MBP fused to His <sub>6</sub> -tagged amino acids 303-329 of Bc TssA1 <sup>B</sup> (includes $\alpha$ -helix H12). <i>B. cenocepacia</i> DNA was amplified with primers pACYC-tssA.CTDfor and pACYC-tssA.CTDH1rev2, the product was restricted with <i>NdeI</i> and <i>BamHI</i> , and ligated between the same sites of pMAL-c5X.       | This study |
| pMAL-c5X-His <sub>6</sub> .TssA1 <sup>B</sup> .CTD H12-H13 | pMAL-c5X encoding MBP fused to His <sub>6</sub> -tagged amino acids 303-347 of Bc TssA1 <sup>B</sup> (includes $\alpha$ -helices H12-H13). <i>B. cenocepacia</i> DNA was amplified with primers pACYC-tssA.CTDfor and pACYC-tssA.CTDH2rev2, the product was restricted with <i>NdeI</i> and <i>BamHI</i> , and ligated between the same sites of pMAL-c5X. | This study |
| pMAL-c5X-His <sub>6</sub> .TssA1 <sup>B</sup> .CTD H12-H14 | pMAL-c5X encoding MBP fused to His <sub>6</sub> -tagged amino acids 303-358 of Bc TssA1 <sup>B</sup> (includes $\alpha$ -helices H12-H14). <i>B. cenocepacia</i> DNA was amplified with primers pACYC-tssA.CTDfor and pACYC-tssA.CTDH3rev2, the product was restricted with <i>NdeI</i> and <i>BamHI</i> , and ligated between the same sites of pMAL-c5X. | This study |
| pMAL-c5X-His <sub>6</sub> .TssA1 <sup>B</sup> .CTD         | pMAL-c5X encoding MBP fused to His <sub>6</sub> -tagged amino acids 303-373 of Bc TssA1 <sup>B</sup> , i.e. the entire CTD (includes H12-H15). <i>B. cenocepacia</i> DNA was amplified with primers pACYC-tssA.CTDfor and pET14b-iotArev, the product was restricted with <i>NdeI</i> and <i>BamHI</i> , and ligated between the same sites of pMAL-c5X.   | This study |
| pET21a-TssA2 <sup>A</sup> .Nt1-Nt2.His <sub>6</sub>        | pET21a encoding Nt1-Nt2 region of TssA2 <sup>A</sup> with His-tag at C-terminus of Nt2. <i>A. hydrophila</i> tssA codons (1-374) amplified with primers pET21a-                                                                                                                                                                                            | This study |

|                                                     |                                                                                                                                                                                                                                                                                                                                                                                                     |            |
|-----------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
|                                                     | AHA1844.Nt1Nt2.for and pET21a-AHA1844.Nt1Nt2.rev, and ligated between the <i>NdeI</i> and <i>XhoI</i> sites of pET21a                                                                                                                                                                                                                                                                               |            |
| pACYCDuet-His <sub>6</sub> .TssA2 <sup>A</sup> .CTD | pACYCDuet-1 encoding N-terminal His-tagged TssA2 <sup>A</sup> CTD. <i>A. hydrophila</i> <i>tssA</i> codons 381-478 amplified with primers pACYC-AHA1844.CTDS2for and pACYC-AHA1844.rev, and ligated between the <i>Bam</i> HI and <i>Hind</i> III sites of pACYCDuet-1                                                                                                                              | This study |
| pET14b-His <sub>6</sub> .TssD2                      | pET14b encoding N-terminal His-tagged TssD. <i>B. cenocepacia</i> <i>tssD</i> amplified with primers pET-His-iotDfor2 and pET14b-iotDrev, cut with <i>NdeI</i> and <i>Bam</i> HI, and ligated to the same sites of pET14b. Expresses TssD lacking the first three amino acids of TssD and containing a 20 amino acid N-terminal tag that harbours a hexahistidine tag and a thrombin cleavage site. | This study |
| <b>Protein expression for co-IP</b>                 |                                                                                                                                                                                                                                                                                                                                                                                                     |            |
| pACYCDuet-FLAG.tssA                                 | <i>B. cenocepacia</i> <i>tssA</i> amplified with primers TssA.NdeI.FLAG.for and TssA.BglII.rev, and inserted between <i>NdeI</i> and <i>Bgl</i> II sites of pACYCDuet-1. Expresses FLAG.TssA1 <sup>B</sup> .                                                                                                                                                                                        | This study |
| pACYCDuet-tssC.VSVg                                 | <i>B. cenocepacia</i> <i>tssC</i> amplified with primers tssC.NcoI.for and tssC.VSVg.BamHI.rev, and inserted between <i>NcoI</i> and <i>Bam</i> HI sites of pACYCDuet-1. Expresses TssC.VSVg.                                                                                                                                                                                                       | This study |
| pACYCDuet-tssC.VSVg-FLAG.tssA                       | <i>B. cenocepacia</i> <i>tssC</i> amplified with primers tssC.NcoI.for and tssC.VSVg.BamHI.rev, and inserted between <i>NcoI</i> and <i>Bam</i> HI sites of pACYCDuet-FLAG.tssA. Expresses TssC.VSVg and FLAG.TssA1 <sup>B</sup> .                                                                                                                                                                  | This study |
| pACYCDuet-VSVg.tssD                                 | <i>B. cenocepacia</i> <i>tssD</i> amplified with tssD.forpACYC.NtermVSVgTag and tssD.BamHI.rev, and inserted between <i>NcoI</i> and                                                                                                                                                                                                                                                                | This study |

|                                                   |                                                                                                                                                                                                                                                                                                                     |            |
|---------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
|                                                   | <i>Bam</i> HI sites of pACYCDuet-1. Expresses <i>vsvg</i> .TssD.                                                                                                                                                                                                                                                    |            |
| pACYCDuet-VSVg.tssD-FLAG.tssA                     | <i>B. cenocepacia tssD</i> amplified with <i>tssD.for</i> pACYC.NtermVSVgTag and <i>tssD</i> . <i>Bam</i> HI.rev, and inserted between <i>Nco</i> I and <i>Bam</i> HI sites of pACYCDuet-FLAG.tssA. Expresses <i>vsvg</i> .TssD and <i>FLAG</i> .TssA1 <sup>B</sup> .                                               | This study |
| pMAL-c5X-His <sub>6</sub> .tssE                   | <i>B. cenocepacia tssE</i> amplified with pMALtssEfor and pMALtssErev, and inserted between <i>Nde</i> I and <i>Bam</i> HI sites of pMAL-c5X-His <sub>6</sub> . Expresses His <sub>6</sub> .MBP-TssE.                                                                                                               | This study |
| pACYCDuet-tssF.HA                                 | <i>B. cenocepacia tssF</i> amplified with <i>tssF.Nco</i> I.for and <i>tssF.HA.Hind</i> III.rev, and inserted between <i>Nco</i> I and <i>Hind</i> III sites of pACYCDuet-1. Expresses TssF <sub>.HA</sub> .                                                                                                        | This study |
| pACYCDuet-tssF.HA-FLAG.tssA                       | <i>B. cenocepacia tssF</i> amplified with <i>tssF.Nco</i> I.for and <i>tssF.HA.Hind</i> III.rev, and inserted between <i>Nco</i> I and <i>Hind</i> III sites of pACYCDuet-FLAG.TssA. Expresses TssF <sub>.HA</sub> and <i>FLAG</i> .TssA1 <sup>B</sup> .                                                            | This study |
| pETDuetΔO-HA.tssF                                 | <i>B. cenocepacia tssF</i> amplified with <i>tssF.Nde</i> I.HA.for and C-term-iotFrev, and inserted between <i>Nde</i> I and <i>Acc</i> 65I sites of pETDuetΔO. Expresses HA.TssF.                                                                                                                                  | This study |
| pACYCDuet-VSVg.tssI <sub>gp27gp5</sub>            | <i>B. cenocepacia tssI</i> conserved core region amplified with <i>tssI.Bsp</i> HI.VSVgtag.For and BCAM0148gp5.BglII.rev, and inserted between <i>Nco</i> I and <i>Bam</i> HI sites of pACYCDuet-1. Expresses <i>vsvg</i> .TssI <sub>C</sub> corresponding to the gp27gp5 core region (N-terminal 526 amino acids). | This study |
| pACYCDuet-VSVg.tssI <sub>gp27gp5</sub> -FLAG.tssA | <i>B. cenocepacia tssA</i> amplified with TssA.NdeI.FLAG.for and TssA.BglII.rev, and inserted between <i>Nde</i> I and <i>Bgl</i> II sites of pACYCDuet-VSVg. <i>tssI</i> <sub>gp27gp5</sub> . Expresses <i>vsvg</i> .TssI <sub>C</sub> and <i>FLAG</i> .TssA1 <sup>B</sup> .                                       | This study |

|                                               |                                                                                                                                                                                                                                                                                      |            |
|-----------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| pACYCDuet-VSVg.tssK                           | <i>B. cenocepacia</i> <i>tssK</i> amplified with TssKforpET.NtermVSVgTag and TssK.BglII.rev, and inserted between <i>NcoI</i> and <i>BamHI</i> sites of pACYCDuet-1. Expresses <sub>VSVg</sub> .TssK.                                                                                | This study |
| pACYCDuet-VSVg.tssK-FLAG.tssA                 | <i>B. cenocepacia</i> <i>tssA</i> amplified with TssA.NdeI.FLAG.for and TssA.BglII.rev, and inserted between <i>NdeI</i> and <i>BglII</i> sites of pACYCDuet-VSVg.tssK. Expresses <sub>VSVg</sub> .TssK and <sub>FLAG</sub> .TssA1 <sup>B</sup> .                                    | This study |
| pACYCDuet-VSVg.tssL                           | <i>B. cenocepacia</i> <i>tssL</i> amplified with TssLforpET.NtermVSVgTag and TssL.Rev, and inserted between <i>NcoI</i> and <i>BamHI</i> sites of pACYCDuet-1. Expresses <sub>VSVg</sub> .TssL.                                                                                      | This study |
| pACYCDuet-VSVg.tssL-FLAG.tssA                 | <i>B. cenocepacia</i> <i>tssL</i> amplified with TssLforpET.NtermVSVgTag and TssL.Rev, and inserted between <i>NcoI</i> and <i>BamHI</i> sites of pACYCDuet-FLAG.TssA. Expresses <sub>VSVg</sub> .TssL and <sub>FLAG</sub> .TssA1 <sup>B</sup> .                                     | This study |
| pACYCDuet-VSVg.tssM <sub>NTR</sub>            | <i>B. cenocepacia</i> <i>tssM</i> codons 62-449 amplified with TssM1.VSVg.NcoI.for and TssM1.HindIII.rev, and inserted between <i>NcoI</i> and <i>HindIII</i> sites of pACYCDuet-1. Expresses <sub>VSVg</sub> .TssM <sub>NTR</sub> (TssM cytoplasmic component).                     | This study |
| pACYCDuet-VSVg.tssM <sub>NTR</sub> -FLAG.tssA | <i>B. cenocepacia</i> <i>tssM</i> codons 62-449 amplified with TssM1.VSVg.NcoI.for and TssM1.HindIII.rev, and inserted between <i>NcoI</i> and <i>HindIII</i> sites of pACYCDuet-FLAG.tssA. Expresses <sub>VSVg</sub> .TssM <sub>NTR</sub> and <sub>FLAG</sub> .TssA1 <sup>B</sup> . | This study |
| <b>Two-hybrid system<sup>b</sup></b>          |                                                                                                                                                                                                                                                                                      |            |
| pKT25                                         | Vector for generating fusions to C-terminus of CyaA T25 fragment, <i>ori</i> <sub>p15A</sub> (Km <sup>R</sup> )                                                                                                                                                                      | (15)       |
| pKT25- <i>zip</i>                             | pKT25 containing the coding sequence of the yeast GCN4 leucine zipper fused to the T25 coding sequence                                                                                                                                                                               | (9)        |
| pKNT25                                        | Vector for generating fusions to N-terminus of CyaA T25 fragment, <i>ori</i> <sub>p15A</sub> (Km <sup>R</sup> )                                                                                                                                                                      | (16)       |

|                                         |                                                                                                                                                                              |            |
|-----------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| pUT18                                   | Vector for generating fusions to N-terminus of CyaA T18 fragment, <i>ori<sub>pMB1</sub></i> (Ap <sup>R</sup> )                                                               | (15)       |
| pUT18C                                  | Vector for generating fusions to C-terminus of CyaA T18 fragment, <i>ori<sub>pMB1</sub></i> (Ap <sup>R</sup> )                                                               | (15)       |
| pUT18C-zip                              | pUT18C containing the coding sequence of the yeast GCN4 leucine zipper fused to the T18 coding sequence                                                                      | (15)       |
| pKT25-tssA1 <sup>B</sup>                | <i>B. cenocepacia</i> <i>tssA</i> amplified with Cterm-iotAfor and Cterm-iotArev, and inserted between the <i>Pst</i> I and <i>Xba</i> I sites of pKT25.                     | This study |
| pKNT25-tssA1 <sup>B</sup>               | <i>B. cenocepacia</i> <i>tssA</i> amplified with Nterm-iotAfor and N-iotAfullrev, and inserted between the <i>Hind</i> III and <i>Bam</i> HI sites of pKNT25                 | This study |
| pUT18-tssA1 <sup>B</sup>                | <i>B. cenocepacia</i> <i>tssA</i> amplified with Nterm-iotAfor and N-iotAfullrev, and inserted between the <i>Hind</i> III and <i>Bam</i> HI sites of pUT18                  | This study |
| pUT18C-tssA1 <sup>B</sup>               | <i>B. cenocepacia</i> <i>tssA</i> amplified with Cterm-iotAfor and Cterm-iotArev, and inserted between the <i>Pst</i> I and <i>Xba</i> I sites of pUT18C                     | This study |
| pKT25-tssA1 <sup>B<sub>Nt1</sub></sup>  | <i>B. cenocepacia</i> <i>tssA</i> codons 1-256 amplified with C-term-iotAfor and C-iotAlongNTDrev, and inserted between the <i>Pst</i> I and <i>Xba</i> I sites of pKT25     | This study |
| pKNT25-tssA1 <sup>B<sub>Nt1</sub></sup> | <i>B. cenocepacia</i> <i>tssA</i> codons 1-256 amplified with Nterm-iotAfor and N-iotAlongNTDrev, and inserted between the <i>Hind</i> III and <i>Bam</i> HI sites of pKNT25 | This study |
| pUT18-tssA1 <sup>B<sub>Nt1</sub></sup>  | <i>B. cenocepacia</i> <i>tssA</i> codons 1-256 amplified with Nterm-iotAfor and N-iotAlongNTDrev, and inserted between the <i>Hind</i> III and <i>Bam</i> HI sites of pUT18  | This study |
| pUT18C-tssA1 <sup>B<sub>Nt1</sub></sup> | <i>B. cenocepacia</i> <i>tssA</i> codons 1-256 amplified with C-term-iotAfor and C-iotAlongNTDrev, and inserted between the <i>Pst</i> I and <i>Xba</i> I sites of pUT18C    | This study |

|                                          |                                                                                                                                                                          |            |
|------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| pKT25-tssA1 <sup>B</sup> <sub>CTD</sub>  | <i>B. cenocepacia</i> tssA codons 294-374 amplified with C-iotAshortCTDfor and Cterm-iotArev, and inserted between the <i>Pst</i> I and <i>Xba</i> I sites of pKT25      | This study |
| pKNT25-tssA1 <sup>B</sup> <sub>CTD</sub> | <i>B. cenocepacia</i> tssA codons 294-373 amplified with N-iotAshortCTDfor and N-iotAfullrev, and inserted between the <i>Hind</i> III and <i>Bam</i> HI sites of pKNT25 | This study |
| pUT18-tssA1 <sup>B</sup> <sub>CTD</sub>  | <i>B. cenocepacia</i> tssA codons 294-373 amplified with N-iotAshortCTDfor and N-iotAfullrev, and inserted between the <i>Hind</i> III and <i>Bam</i> HI sites of pUT18  | This study |
| pUT18C-tssA1 <sup>B</sup> <sub>CTD</sub> | <i>B. cenocepacia</i> tssA codons 294-374 amplified with C-iotAshortCTDfor and Cterm-iotArev, and inserted between the <i>Pst</i> I and <i>Xba</i> I sites of pUT18C     | This study |
| pKT25-tssA2 <sup>A</sup>                 | <i>A. hydrophila</i> tssA amplified with primers AHA1844fullfor and AHA1844-Crev2, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pKT25.                | This study |
| pKNT25-tssA2 <sup>A</sup>                | <i>A. hydrophila</i> tssA amplified with primers AHA1844fullfor and AHA1844-Nrev, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pKNT25                 | This study |
| pUT18-tssA2 <sup>A</sup>                 | <i>A. hydrophila</i> tssA amplified with primers AHA1844fullfor and AHA1844-Nrev, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pUT18                  | This study |
| pUT18C-tssA2 <sup>A</sup>                | <i>A. hydrophila</i> tssA amplified with primers AHA1844fullfor and AHA1844-Crev2, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pUT18C.               | This study |
| pKT25-tssA2 <sup>A</sup> Nt1             | <i>A. hydrophila</i> tssA codons 1-229 amplified with primers AHA1844fullfor and AHA1844NTDrev, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pKT25    | This study |
| pKNT25-tssA2 <sup>A</sup> Nt1            | <i>A. hydrophila</i> tssA codons 1-229 amplified with primers AHA1844fullfor and AHA1844NTDrev2,                                                                         | This study |

|                                   |                                                                                                                                                                        |            |
|-----------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
|                                   | and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pKNT25                                                                                                 |            |
| pUT18-tssA2 <sup>A</sup> Nt1      | <i>A. hydrophila</i> tssA codons 1-229 amplified with primers AHA1844fullfor and AHA1844NTDrev2, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pUT18 | This study |
| pUT18C-tssA2 <sup>A</sup> Nt1     | <i>A. hydrophila</i> tssA codons 1-229 amplified with primers AHA1844fullfor and AHA1844NTDrev, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pUT18C | This study |
| pKT25-tssA2 <sup>A</sup> Nt2      | <i>A. hydrophila</i> tssA codons 244-386 amplified with primers AHA1844for and AHA1844CTDrev, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pKT25    | This study |
| pUT18C-tssA2 <sup>A</sup> Nt2     | <i>A. hydrophila</i> tssA codons 244-386 amplified with primers AHA1844for and AHA1844CTDrev, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pUT18C   | This study |
| pKT25-tssA2 <sup>A</sup> CTD      | <i>A. hydrophila</i> tssA codons 381-478 amplified with primers AHA1844for2 and AHA1844-Crev2, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pKT25   | This study |
| pUT18C-tssA2 <sup>A</sup> CTD     | <i>A. hydrophila</i> tssA codons 381-478 amplified with primers AHA1844for2 and AHA1844-Crev2, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pUT18C  | This study |
| pKT25-tssA2 <sup>A</sup> Nt2-CTD  | <i>A. hydrophila</i> tssA codons 244-478 amplified with primers AHA1844for and AHA1844-Crev2, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pKT25    | This study |
| pUT18C-tssA2 <sup>A</sup> Nt2-CTD | <i>A. hydrophila</i> tssA codons 244-478 amplified with primers AHA1844for and AHA1844-Crev2, and inserted between the <i>Xba</i> I and <i>Kpn</i> I sites of pUT18C   | This study |

|             |                                                                                                                                                                                                                                        |            |
|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| pKT25-tssB  | <i>B. cenocepacia</i> <i>tssB</i> amplified with Cterm-iotBfor and Cterm-iotBrev, and inserted between the <i>Xba</i> I and <i>Eco</i> RI sites of pKT25                                                                               | This study |
| pKNT25-tssB | <i>B. cenocepacia</i> <i>tssB</i> amplified with Nterm-iotBfor and N-iotBfullrev, and inserted between the <i>Sph</i> I and <i>Xba</i> I sites of pKNT25                                                                               | This study |
| pUT18-tssB  | <i>B. cenocepacia</i> <i>tssB</i> amplified with Nterm-iotBfor and N-iotBfullrev, and inserted between <i>Sph</i> I and <i>Xba</i> I sites of pUT18                                                                                    | This study |
| pUT18C-tssB | <i>B. cenocepacia</i> <i>tssB</i> amplified with Cterm-iotBfor and Cterm-iotBrev, and inserted between the <i>Xba</i> I and <i>Eco</i> RI sites of pUT18C                                                                              | This study |
| pKT25-tssC  | <i>B. cenocepacia</i> <i>tssC</i> amplified with Cterm-iotCfor and Cterm-iotCrev, and inserted between the <i>Pst</i> I and <i>Xba</i> I sites of pKT25                                                                                | This study |
| pKNT25-tssC | <i>B. cenocepacia</i> <i>tssC</i> amplified with Nterm-iotCfor and Nterm-tssCrev.new, and inserted between the <i>Hind</i> III and <i>Bam</i> HI sites of pKNT25, followed by <i>Acc</i> 65I digestion, end filling and self-ligation. | This study |
| pUT18-tssC  | <i>B. cenocepacia</i> <i>tssC</i> amplified with Nterm-iotCfor and Nterm-tssCrev.new, and inserted between <i>Hind</i> III and <i>Bam</i> HI sites of pUT18                                                                            | This study |
| pUT18C-tssC | <i>B. cenocepacia</i> <i>tssC</i> amplified with Cterm-tssCfor.new and Cterm-iotCrev, and inserted between the <i>Pst</i> I and <i>Xba</i> I sites of pUT18C                                                                           | This study |
| pKT25-tssD  | <i>B. cenocepacia</i> <i>tssD</i> amplified with pUT18C-iotDfor and Cterm-iotDrev, and inserted between the <i>Pst</i> I and <i>Xba</i> I sites of pKT25                                                                               | This study |
| pKNT25-tssD | <i>B. cenocepacia</i> <i>tssD</i> amplified with Nterm-iotDfor and Nterm-iotDrev, and inserted between the <i>Hind</i> III and <i>Bam</i> HI sites of pKNT25                                                                           | This study |
| pUT18-tssD  | <i>B. cenocepacia</i> <i>tssD</i> amplified with Nterm-iotDfor and Nterm-iotDrev, and inserted between <i>Hind</i> III and <i>Bam</i> HI sites of pUT18                                                                                | This study |

|             |                                                                                                                                                                      |            |
|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| pUT18C-tssD | <i>B. cenocepacia</i> <i>tssD</i> amplified with pUT18C-<br>iotDfor and Cterm-iotDrev, and inserted between<br>the <i>Pst</i> I and <i>Xba</i> I sites of pUT18C     | This study |
| pKT25-tssE  | <i>B. cenocepacia</i> <i>tssE</i> amplified with Cterm-iotEfor<br>and Cterm-iotErev, and inserted between the<br><i>Xba</i> I and <i>Eco</i> RI sites of pKT25       | This study |
| pKNT25-tssE | <i>B. cenocepacia</i> <i>tssE</i> amplified with Nterm-iotEfor<br>and N-iotEfullrev, and inserted between the<br><i>Hind</i> III and <i>Bam</i> HI sites of pKNT25   | This study |
| pUT18-tssE  | <i>B. cenocepacia</i> <i>tssE</i> amplified with Nterm-iotEfor<br>and N-iotEfullrev, and inserted between <i>Hind</i> III<br>and <i>Bam</i> HI sites of pUT18        | This study |
| pUT18C-tssE | <i>B. cenocepacia</i> <i>tssE</i> amplified with Cterm-iotEfor<br>and Cterm-iotErev, and inserted between the<br><i>Xba</i> I and <i>Eco</i> RI sites of pUT18C      | This study |
| pKT25-tssF  | <i>B. cenocepacia</i> <i>tssF</i> amplified with Cterm-iotFfor<br>and Cterm-iotFrev, and inserted between the <i>Xba</i> I<br>and <i>Acc</i> 65I sites of pKT25      | This study |
| pKNT25-tssF | <i>B. cenocepacia</i> <i>tssF</i> amplified with Nterm-iotFfor<br>and N-iotFfullrev, and inserted between the<br><i>Hind</i> III and <i>Xba</i> I sites of pKNT25    | This study |
| pUT18-tssF  | <i>B. cenocepacia</i> <i>tssF</i> amplified with Nterm-iotFfor<br>and N-iotFfullrev, and inserted between <i>Hind</i> III<br>and <i>Xba</i> I sites of pUT18         | This study |
| pUT18C-tssF | <i>B. cenocepacia</i> <i>tssF</i> amplified with Cterm-iotFfor<br>and Cterm-iotFrev, and inserted between the <i>Xba</i> I<br>and <i>Acc</i> 65I sites of pUT18C     | This study |
| pKT25-tssG  | <i>B. cenocepacia</i> <i>tssG</i> amplified with C-<br>iotGfrag1for and Cterm-iotGrev, and inserted<br>between the <i>Xba</i> I and <i>Eco</i> RI sites of pKT25     | This study |
| pKNT25-tssG | <i>B. cenocepacia</i> <i>tssG</i> amplified with N-iotGfrag1for<br>and Nterm-iotGrev2, and inserted between the<br><i>Hind</i> III and <i>Bam</i> HI sites of pKNT25 | This study |
| pUT18-tssG  | <i>B. cenocepacia</i> <i>tssG</i> amplified with N-iotGfrag1for<br>and Nterm-iotGrev2, and inserted between <i>Hind</i> III<br>and <i>Bam</i> HI sites of pUT18      | This study |

|                          |                                                                                                                                                                                                                                   |            |
|--------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| pUT18C-tssG              | <i>B. cenocepacia</i> <i>tssG</i> amplified with C-iotGfrag1for and Cterm-iotGrev, and inserted between the <i>Xba</i> I and <i>Eco</i> RI sites of pUT18C                                                                        | This study |
| pKT25-tssI               | <i>B. cenocepacia</i> <i>tssI</i> amplified with TssIfor and C-tssIrev, and inserted between the <i>Xba</i> I and <i>Bam</i> HI sites of pKT25 following cleavage of the amplicon with <i>Xba</i> I and <i>Bgl</i> II             | This study |
| pKNT25-tssI              | <i>B. cenocepacia</i> <i>tssI</i> amplified with TssIfor and N-tssIrev, and inserted between the <i>Xba</i> I and <i>Bam</i> HI of pKNT25 following cleavage of the amplicon with <i>Xba</i> I and <i>Bgl</i> II                  | This study |
| pUT18-tssI               | <i>B. cenocepacia</i> <i>tssI</i> amplified with TssIfor and N-tssIrev, and inserted between the <i>Xba</i> I and <i>Bam</i> HI sites of pUT18 following cleavage of the amplicon with <i>Xba</i> I and <i>Bgl</i> II             | This study |
| pUT18C-tssI              | <i>B. cenocepacia</i> <i>tssI</i> amplified with TssIfor and C-tssIrev, and inserted between the <i>Xba</i> I and <i>Bam</i> HI sites of pUT18C following cleavage of the amplicon with <i>Xba</i> I and <i>Bgl</i> II            | This study |
| pKT25-tssI <sub>C</sub>  | <i>B. cenocepacia</i> <i>tssI</i> codons 1-526 amplified with TssIfor and Rev.gp5, and inserted between the <i>Xba</i> I and <i>Bam</i> HI sites of pKT25 following cleavage of the amplicon with <i>Xba</i> I and <i>Bgl</i> II  | This study |
| pUT18C-tssI <sub>C</sub> | <i>B. cenocepacia</i> <i>tssI</i> codons 1-526 amplified with TssIfor and Rev.gp5, and inserted between the <i>Xba</i> I and <i>Bam</i> HI sites of pUT18C following cleavage of the amplicon with <i>Xba</i> I and <i>Bgl</i> II | This study |
| pKT25-tssJ               | <i>B. cenocepacia</i> <i>tssJ</i> codons 13-200 amplified with For1tssj and C-tssJrev, and inserted between the <i>Pst</i> I and <i>Bam</i> HI sites of pKT25                                                                     | This study |
| pKNT25-tssJ              | <i>B. cenocepacia</i> <i>tssJ</i> codons 13-200 amplified with For2tssj and N-tssJrev, and inserted between the <i>Pst</i> I and <i>Bam</i> HI sites of pKNT25                                                                    | This study |
| pUT18-tssJ               | <i>B. cenocepacia</i> <i>tssJ</i> codons 13-200 amplified with For2tssj and N-tssJrev, and inserted between <i>Pst</i> I and <i>Bam</i> HI sites of pUT18                                                                         | This study |

|                            |                                                                                                                                                                                                                                    |            |
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| pUT18C-tssJ                | <i>B. cenocepacia</i> <i>tssJ</i> codons 13-200 amplified with For2tssj and C-tssJrev, and inserted between the <i>Pst</i> I and <i>Bam</i> HI sites of pUT18C                                                                     | This study |
| pKT25-tssK                 | <i>B. cenocepacia</i> <i>tssK</i> amplified with tssKfor and C-tssKrev, and inserted between the <i>Bam</i> HI and <i>Acc</i> 65I sites of pKT25                                                                                   | This study |
| pKNT25-tssK                | <i>B. cenocepacia</i> <i>tssK</i> amplified with tssKfor and N-tssKrev, and inserted between the <i>Hind</i> III and <i>Bam</i> HI sites of pKNT25                                                                                 | This study |
| pUT18-tssK                 | <i>B. cenocepacia</i> <i>tssK</i> amplified with tssKfor and N-tssKrev, and inserted between <i>Bam</i> HI and <i>Acc</i> 65I sites of pUT18                                                                                       | This study |
| pUT18C-tssK                | <i>B. cenocepacia</i> <i>tssK</i> amplified with tssKfor and C-tssKrev, and inserted between the <i>Bam</i> HI and <i>Acc</i> 65I sites of pUT18C                                                                                  | This study |
| pKT25-tssL                 | <i>B. cenocepacia</i> <i>tssL</i> codons 1-204 amplified with tssLfor and C-tssLrev, and inserted between the <i>Xba</i> I and <i>Acc</i> 65I sites of pKT25                                                                       | This study |
| pKNT25-tssL                | <i>B. cenocepacia</i> <i>tssL</i> codons 1-204 amplified with tssLfor and N-tssLrev, and inserted between the <i>Xba</i> I and <i>Acc</i> 65I sites of pKNT25                                                                      | This study |
| pUT18-tssL                 | <i>B. cenocepacia</i> <i>tssL</i> codons 1-204 amplified with tssLfor and N-tssLrev, and inserted between <i>Xba</i> I and <i>Acc</i> 65I sites of pUT18                                                                           | This study |
| pUT18C-tssL                | <i>B. cenocepacia</i> <i>tssL</i> codons 1-204 amplified with tssLfor and C-tssLrev, and inserted between the <i>Xba</i> I and <i>Acc</i> 65I sites of pUT18C                                                                      | This study |
| pKT25-tssM <sub>NTR</sub>  | <i>B. cenocepacia</i> <i>tssM</i> codons 62-450 amplified with tssM1for and C-tssM1rev, and inserted between the <i>Xba</i> I and <i>Acc</i> 65I sites of pKT25                                                                    | This study |
| pKNT25-tssM <sub>NTR</sub> | <i>B. cenocepacia</i> <i>tssM</i> codons 62-450 amplified with tssM1for and N-tssM1rev, inserted between the <i>Xba</i> I and <i>Acc</i> 65I sites of pKNT25, followed by <i>Acc</i> 65I digestion, end filling and self-ligation. | This study |
| pUT18-tssM <sub>NTR</sub>  | <i>B. cenocepacia</i> <i>tssM</i> codons 62-450 amplified with tssM1for and N-tssM1rev, and inserted                                                                                                                               | This study |

|                            |                                                                                                                                                                     |            |
|----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
|                            | between <i>Xba</i> I and <i>Acc</i> 65I sites of pUT18, followed by <i>Acc</i> 65I digestion, DNA end filling and self-ligation.                                    |            |
| pUT18C-tssM <sub>NTR</sub> | <i>B. cenocepacia</i> <i>tssM</i> codons 62-450 amplified with tssM1for and C-tssM1rev, and inserted between the <i>Xba</i> I and <i>Acc</i> 65I sites of pUT18C    | This study |
| pKT25-tssM <sub>CTR</sub>  | <i>B. cenocepacia</i> <i>tssM</i> codons 467-1309 amplified with tssM2for and C-tssM2rev, and inserted between the <i>Bam</i> HI and <i>Acc</i> 65I sites of pKT25  | This study |
| pKNT25-tssM <sub>CTR</sub> | <i>B. cenocepacia</i> <i>tssM</i> codons 467-1308 amplified with tssM2for and N-tssM2rev, and inserted between the <i>Bam</i> HI and <i>Acc</i> 65I sites of pKNT25 | This study |
| pUT18-tssM <sub>CTR</sub>  | <i>B. cenocepacia</i> <i>tssM</i> codons 467-1308 amplified with tssM2for and N-tssM2rev, and inserted between the <i>Bam</i> HI and <i>Acc</i> 65I sites of pUT18  | This study |
| pUT18C-tssM <sub>CTR</sub> | <i>B. cenocepacia</i> <i>tssM</i> codons 467-1309 amplified with tssM2for and C-tssM2rev, and inserted between the <i>Bam</i> HI and <i>Acc</i> 65I sites of pUT18C | This study |

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<sup>a</sup>Ap<sup>R</sup>, ampicillin resistance; Cm<sup>R</sup>, chloramphenicol resistance; Km<sup>R</sup>, kanamycin resistance; Tp<sup>R</sup>, trimethoprim resistance; BHR, broad host-range

<sup>b</sup>T6SS subunit or domain coding sequences included the native or an added translation termination codon (as appropriate) when ligated to pKT25 or pUT18C but were omitted for pKNT25 and pUT18.

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