TF is a *bona fide* secretory pathway chaperone and interacts with SecB and the translocase

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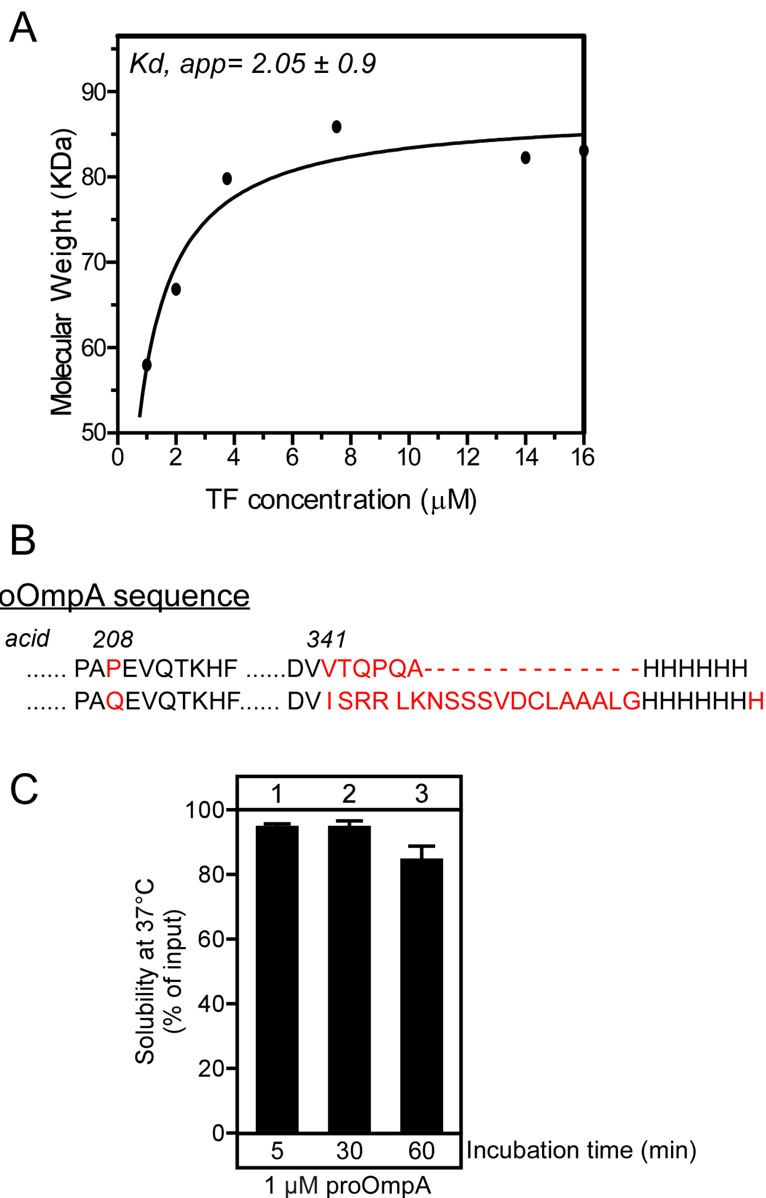
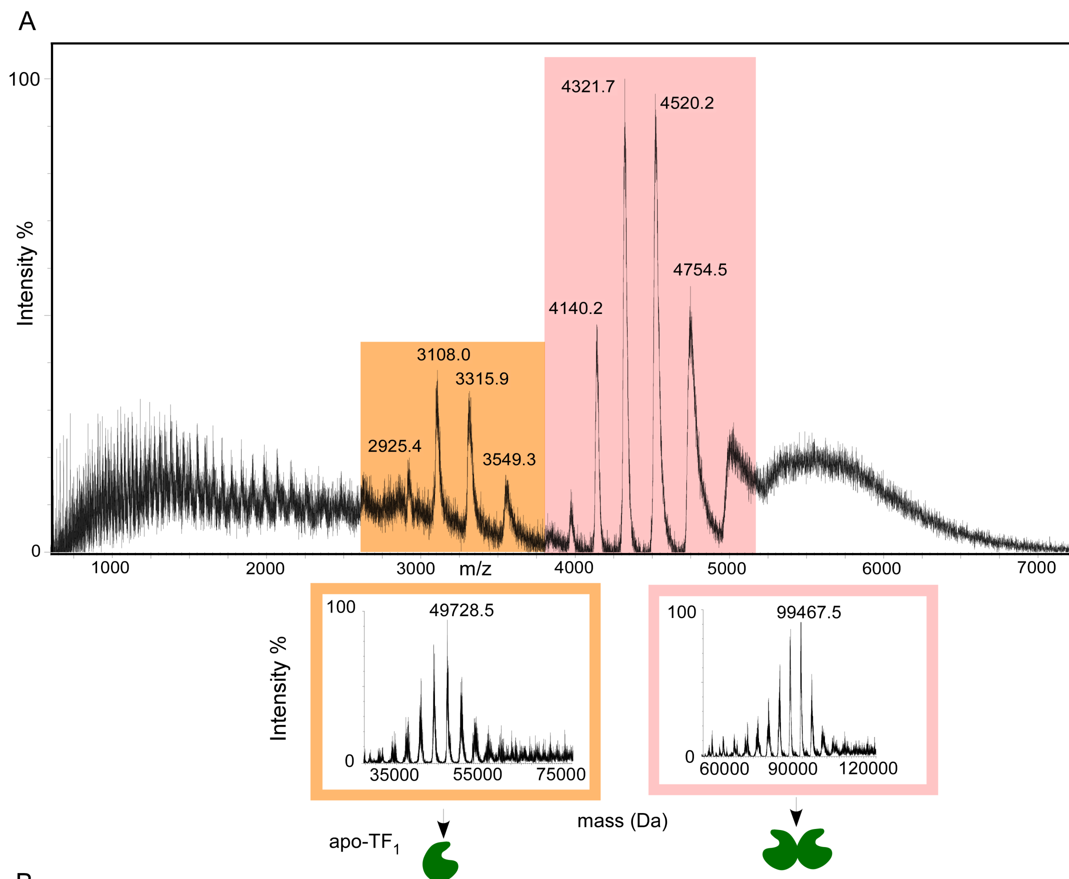


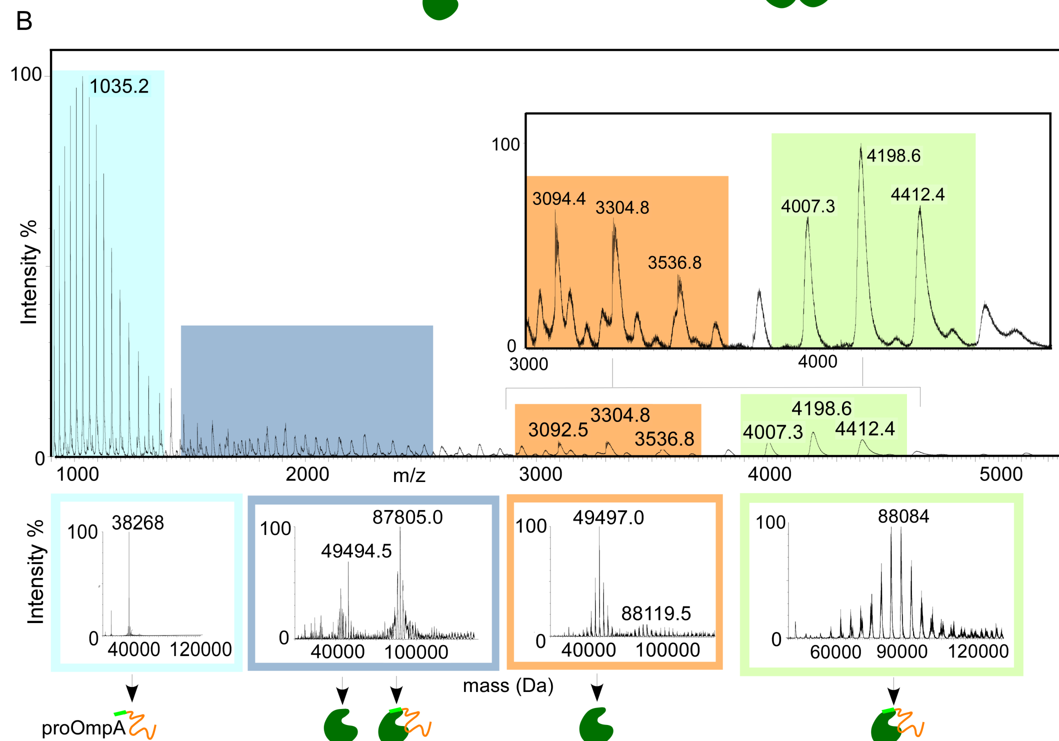
Appendix Figure S1. Apparent K_d of TF dimerization, solubility of proOmpA after pre-incubation times and linear sequence of proOmpA* (related to Figures 1 and 2).

A. Apparent K_d of TF dimerization in solution. The molecular mass of TF was measured using SEC-MALS at the indicated concentration range. Data were plotted using Graph-Pad Prism and the K_d was determined using non-linear regression analysis; *n=1* biological replicate

B. Linear sequence of proOmpA (P0A910) and the proOmpA*derivative. Mutated amino acids indicated in red, (-) amino acid insertion. All mutations and additions reside in the C-terminal periplasmic peptidoglycan-binding domain of proOmpA.

C. proOmpA is retained soluble and does not aggregate at 37°C. $proOmpA_1$ (1 μM) was incubated (Buffer C; < 0.2 M urea; 37°C) as indicated and soluble proteins (harvested after centrifugation; 20,000 x g; 10 min; 4°C) were estimated using Bradford assay (Biorad). The soluble proOmpA remaining in different time points is expressed as a percentage of the input. *n*= 4 biological replicates; mean values ± SEM are shown.

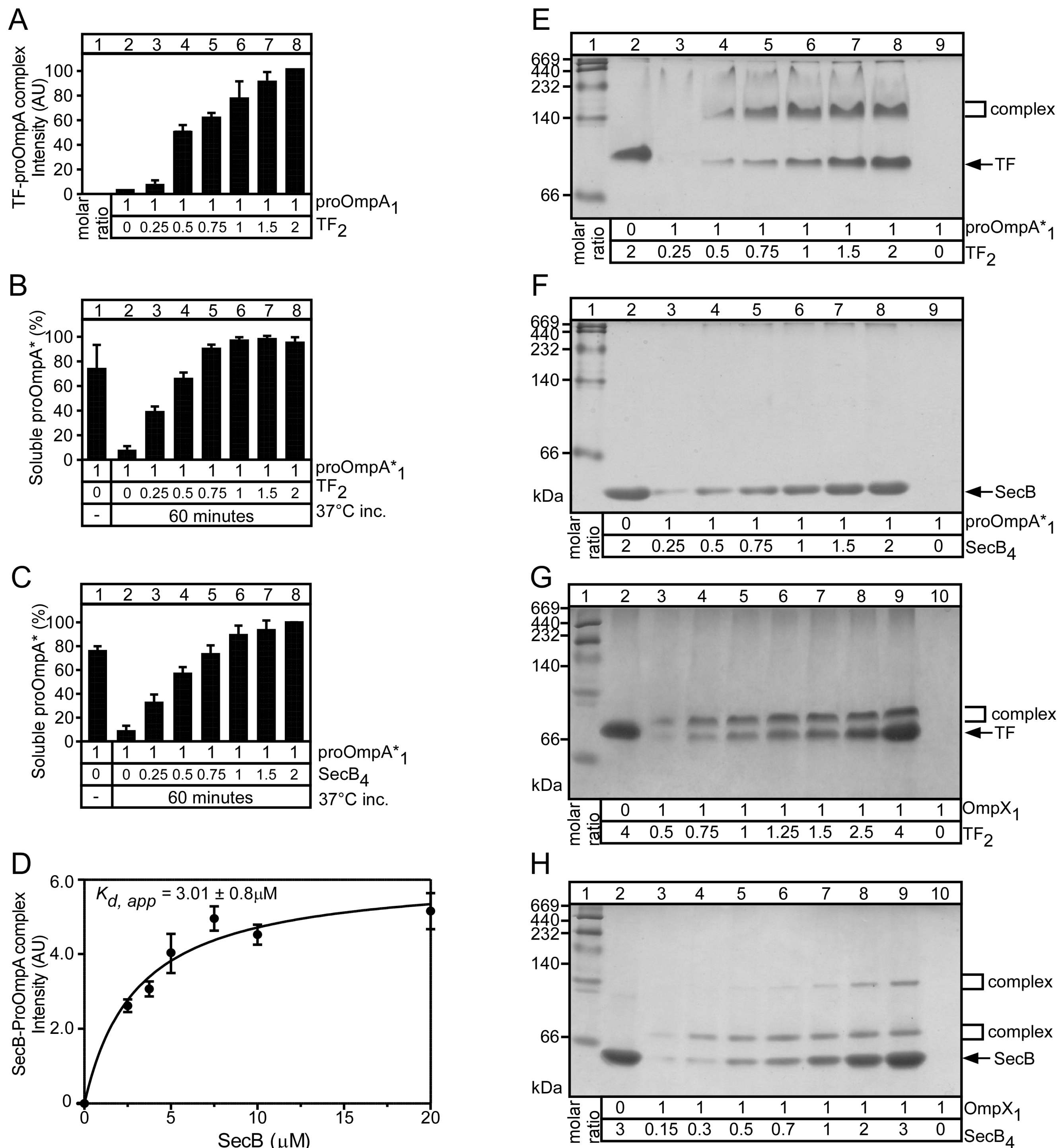




Appendix Figure S2. Native-MS of TF_2 and TF:proOmpA (Related to Fig. 2).

A. TF_2 native-MS analysis at 37°C**.** ESI-mass spectrum and charge state distributions of monomeric (TF_1) and dimeric (TF_2) populations (orange and pink respectively). The deconvoluted mass spectra of native TF_2 are shown (TF_1: 49497.0; Da TF_2:99467.5 Da). Representative experiment is shown; *n=3* biological replicates

B. TF:proOmpA native-MS analysis at 37oC. ESI-mass spectrum and charge state distributions of different protein populations are shown: proOmpA, (TF: proOmpA)$_1$, TF_1 and (TF: proOmpA)$_2$ are colored in cyan, light blue, orange and green respectively. The deconvoluted mass spectra from different populations of the complexes and the derived stoichiometries are shown (cartoon representation). A representative experiment is shown; *n=3* biological replicates



Appendix Figure S3. Quantification of SDS-PAGE gels and native-PAGE analyses of TF and SecB interactions and derived SecB-proOmpA affinity (related to Figures 2 and 4).

A. Quantification of TF: proOmpA complex formed. Signal intensities from Fig. 2A were quantified using Image J software (https://imagej.nih.gov/ij/) and expressed as a percentage of the maximum complex-intensity measured (lane 8); mean values with SEM are shown; *n=4* biological replicates

B. TF rescues proOmpA* from aggregation. Signal intensities from Fig. 2C were quantified using Image J software (https://imagej.nih.gov/ij/) and expressed as percentage of maximum

proOmpA*-intensity measured (lane 9); mean values with SEM are shown; *n=4* biological replicates

C. SecB rescues proOmpA* from aggregation Signal intensities from Fig. 4B were analyzed as in B; mean values with SEM are shown; *n=4* biological replicates

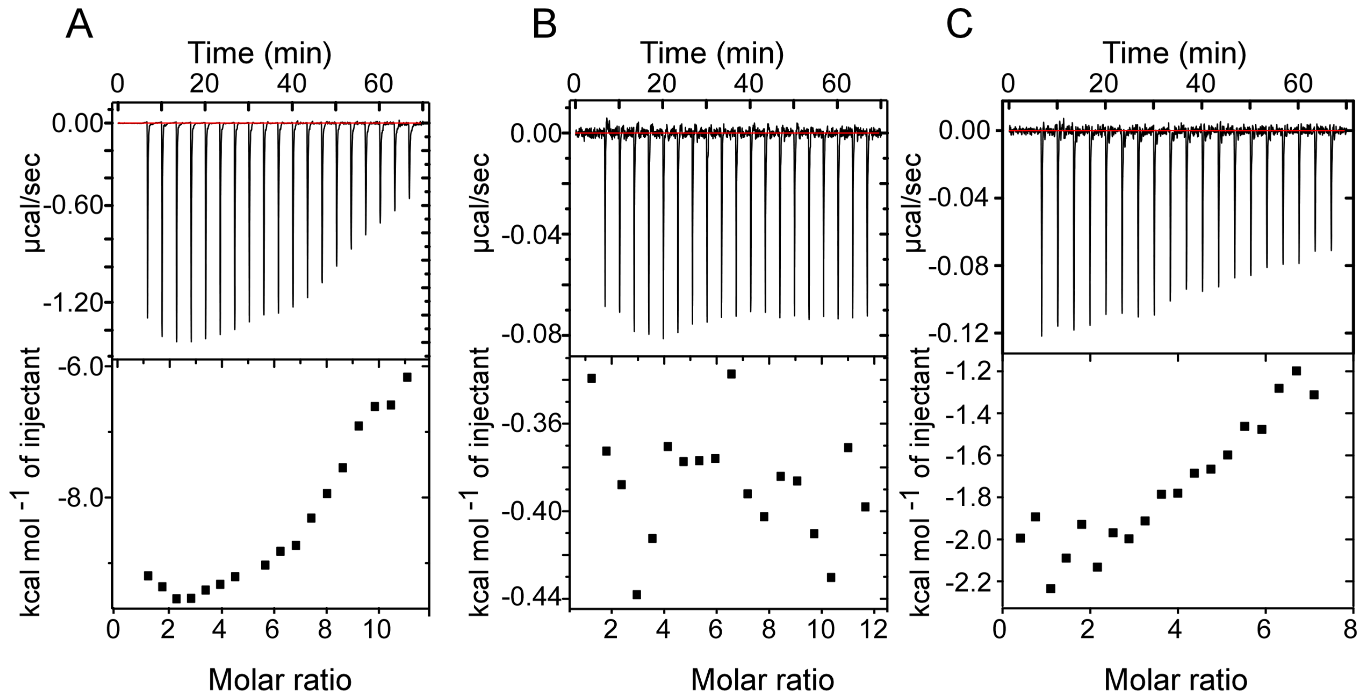
D. Apparent K_d determination of SecB: proOmpA interaction. Signal intensities from Fig. 4C were quantified using Image J software (https://imagej.nih.gov/ij/) and data were plotted on Graph Pad Prism 5.0 as a function of the total concentration of SecB used. *Apparent* K_d was determined after non-linear regression analysis. Mean values with SEM are shown; *n=6* biological replicates

E. TF: proOmpA* complex formation. proOmpA*$_1$ (5 μM; lane 9) was incubated (50 μl; Buffer C; < 0.2 M urea; 37°C; 60 min) with the indicated molar excess of TF_2 (lanes 3-8). Soluble proteins (20,000 x g; 10 min; 4°C) were analyzed on 10% Native-PAGE (4 mA; 16h; 4°C) and Coomassie-blue stained. Lane 2: max TF amount used. Representative experiment is shown; *n*= 4 biological replicates

F. SecB does not form stable complex with proOmpA*. proOmpA*$_1$ was incubated with the indicated molar excess of $SecB_4$ and soluble proteins analyzed as in E. Lane 2: max SecB amount used. Representative experiment is shown; *n*= 4 biological replicates

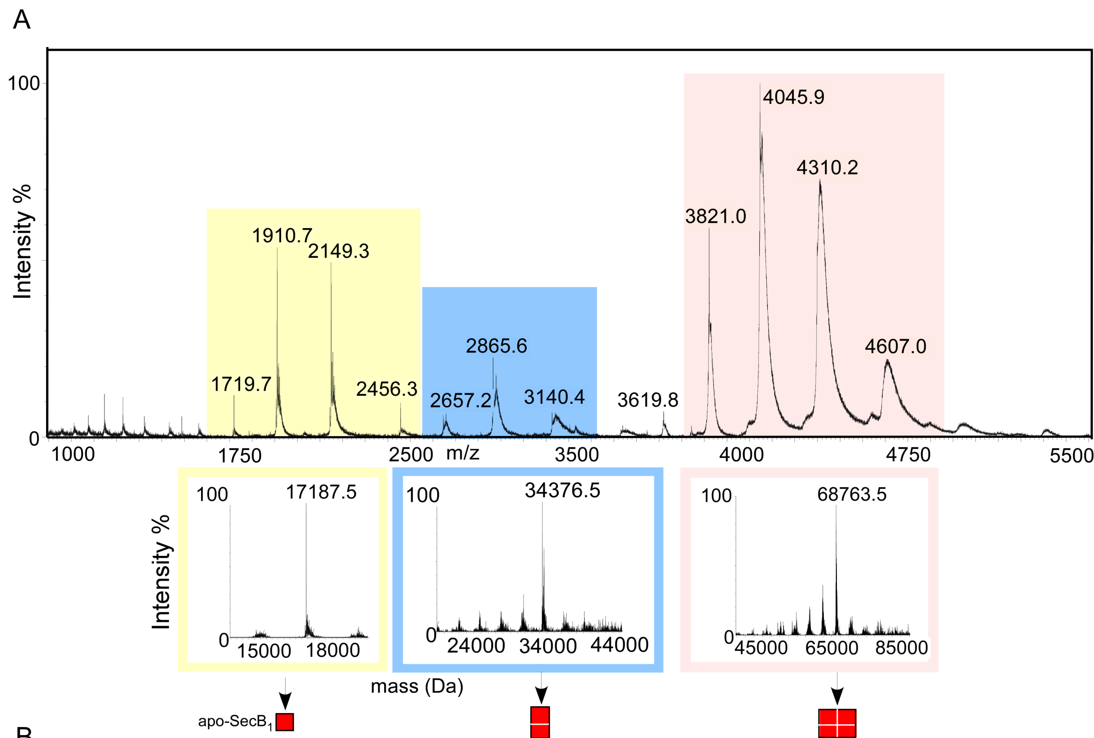
G. TF: proOmpX complex formation. proOmpX$_1$ was incubated with the indicated molar excess of TF_2 and soluble proteins analyzed as in E Lane 2: max TF amount used. Representative experiment is shown; *n*= 4 biological replicates

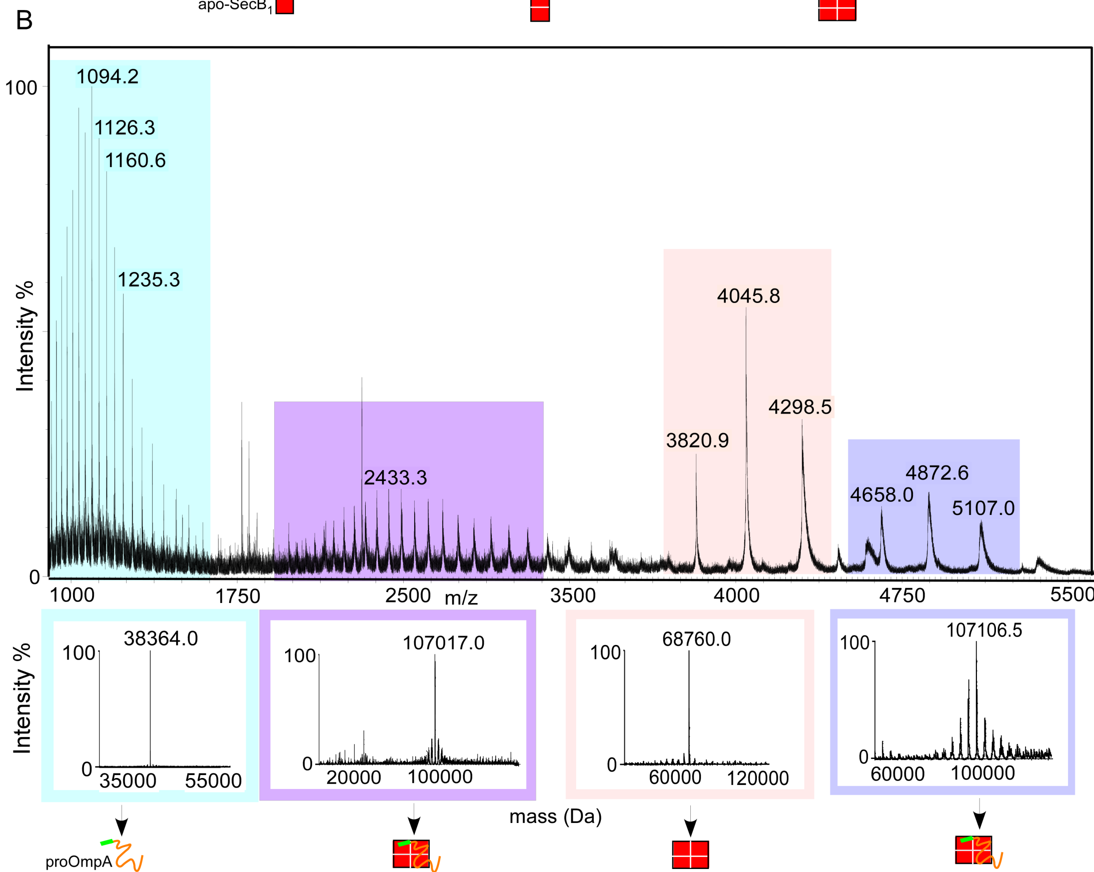
H. SecB: proOmpX complex formation. proOmpX$_1$ was incubated with the indicated molar excess of $SecB_4$ and soluble proteins analyzed as in E Lane 2: max SecB amount used. Representative experiment is shown; *n*= 2 biological replicates



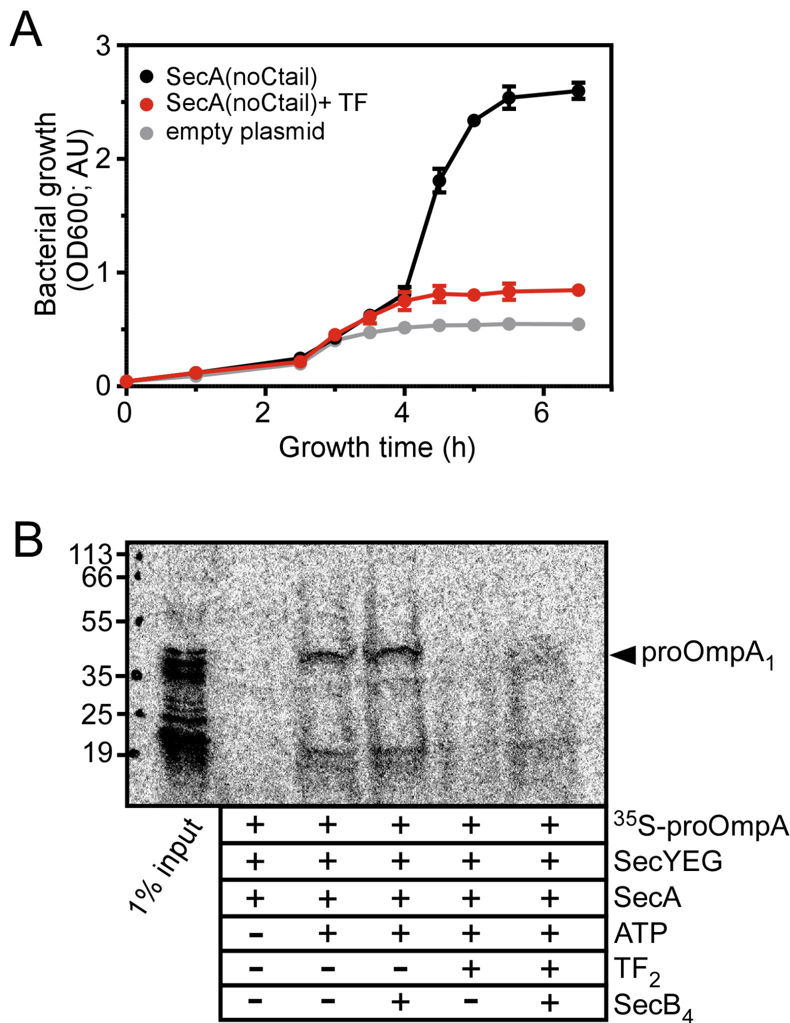
Appendix Figure S4. TF interaction with either SecB, or SecA wt or SecA(noC-tail) in solution (related to Figures 5 and 6).

ITC analysis of TF_2 (cell) with $SecA_2$ **(A)** $SecB_4$ **(B)** and SecA-ΔC-tail **(C)** in the syringe. Experiments were performed in Buffer H at 25°C using an iTC200 instrument (Malvern). The raw data are shown as an injection profile (top) and the calorimetric binding isotherm (bottom). No measurable K_d derived from these experiments. A representative experiment is shown; *n=3* biological replicates





Appendix Figure S5. Native MS of $SecB_4$ and SecB:proOmpA (related to Figures 4 and 5).
A. $SecB_4$ native-MS analysis at 37°C**.** ESI-mass spectrum and charge state distributions of monomer ($SecB_1$), dimer ($SecB_2$) and tetramer ($SecB_4$) populations are colored in yellow, blue and light pink respectively. The deconvoluted mass spectra of native $SecB_4$ are shown ($SecB_1$: 17187.5 Da; SecB2: 34376.5 Da; $SecB_4$: 68763.5 Da). Representative experiment is shown; *n=3* biological replicates **B**. SecB:proOmpA native-MS analysis at 37oC. ESI-mass spectrum and charge state distributions of different protein populations are shown: proOmpA, ($SecB_4$: proOmpA)$_1$, $SecB_4$ and ($SecB_4$: proOmpA)$_2$ are colored in cyan, violet, light pink and light purple respectively. The deconvoluted mass spectra from different populations of the complexes and the derived stoichiometries are shown (cartoon representation). A representative experiment is shown; *n=3* biological replicates



Appendix Figure S6. The C-tail of SecA is not required for TF-mediated inhibition of translocation *in vivo* and proOmpA translocation and chaperone interaction is chaotrope-independent (related to Figure 6)

A. Growth curves of the *E. coli* BL21-19, a strain thermosensitive for the *secA* gene [1], carrying vector pBAD501 (p15A *ori*, gentamycin^R, *ara* promoter)[2] with either the *secA*(no-Ctail) cloned, or empty, and the pASK IBA 7 plus vector (Col E1 ori, ampicillin^R, *tet* promoter) empty or harbouring the cloned *tig* gene (encoding TF), as indicated. Bacteria were grown in LB at 30°C supplemented with Ampicillin (100μg/ml) and Gentamycin (10μg/ml). When culture OD_{600} reached 0.5, gene expression was induced (AHT; 5 ng/ml; L-arabinose; 0.1%w/v), cultures were transferred at 42°C and culture density was determined every 30 minutes post-induction spectrophotometrically. Mean values with SEM are shown; *n*= 3 biological replicates

B. *In vitro* translocation of proOmpA and chaperone effects are independent of chaotrope effects. [^{35}S]-proOmpA was synthesized *in vitro* using a TnT system in the absence of urea, and after removal of the ribosomes by ultra-centrifugation, was used in an *in vitro* SecA-dependent translocation assay (as in Fig. 5C). A representative autoradiograph is shown; *n=3* biological replicates

Appendix Materials

Appendix Table S1: Bacterial Strains

E. coli strain	Description (gene deleted)	Reference/source
MC4100	*F-araD139 ¢(argF-lac)U169 rpsL150 (StrR) relA1 flbB5301 deoC1 pstF25 rbsR*	P. Genevaux [3-5]
MC4100Δ*secB*	In-frame deletion/replacement of the *secB* gene by the *cat* gene.	P. Genevaux [3,4,6,7]
MC4100Δ*tig*Δ*secB*	In-frame deletion/replacement of the *secB* gene by the *cat* gene. The Δ*tig*::Cm^R allele was moved by bacteriophage P1 transduction into MC4100 and the Cm^R cassette was removed by the FLP recombinase.	P. Genevaux [3,4,6,7]
Lemo21(DE3)	T7 RNA polymerase gene under the control of the *lac*UV5 promoter.	New England BioLabs
BL21.19(DE3)	*secA13 (Am) supF (Ts) trp (Am) zch::Tn10 recA::cat clpA::kan)*	[1]
Tuner (DE3)	*lacZY* deletion mutant of BL21.	Novagen
BL31(DE3)	Non *ts* spontaneous revertant of BL21.19(DE3).	[2]

Appendix Table S2: Plasmids

Genes were cloned in the indicated plasmid vectors using combinations of restriction enzymes (as indicated). Mutations were introduced on genes *via* the QuickChange Site-Directed Mutagenesis protocol (Stratagene-Agilent); templates and primers were as indicated. Restriction enzymes and T4 DNA Ligase were either from Minotech (Greece) or Promega or NEB. For PCR mutagenesis PFU Ultra Polymerase (Stratagene) was used; for gene amplification either Expand High fidelity Polymerase (Roche) or PFU Ultra polymerase (Promega) was used. DpnI was used to cleave the maternal methylated DNA (NEB or Promega). All PCR-generated plasmids were sequenced (Macrogen Europe). Plasmids were stored in DH5α cells

Gene	Uniprot accession number	Plasmid name	Vector	Description/source/reference
Preproteins				
pro*ompA**	P0A910	pIMBB454	pET22b	pro*ompA* gene was amplified from pTY009 using the primers X191 and X193 and inserted into the NdeI-EcoRI sites of pET22b. Mutations and additions (Fig. S3A) were introduced during the making of the construct.
pro*ompA*	P0A910	pLMB1835	pET22b	pro*ompA* gene was amplified from pIMBB1589 (pBADpro*ompA*-*phoA*-His) using primers X191 (Forw NdeI) and X1269 (Rev XhoI) and inserted into same sites of pET22b [8].
pro*phoA Δcys*	P00634	pIMBB977	pET22b	[9]
Mature domains				
ompA	P0A910	pIMBB1386	pET22b	*OmpA* gene was amplified from DH5a strain using primers X558 (Forw NdeI)

				and X1269 (Rev XhoI) and inserted to the same sites of pET22b [8].
Chaperones				
secB	P0AG86	pIMBB1602	pASKIBA7 plus	*secB* gene amplified from pIMBB490 (pET16b*secB*) using primers X1944 (Forw XbaI with RBS) and X1945 (Rev HindIII) and was inserted to the same sites of pASKIBA7plus.
tig	P0A850	pIMBB1605	pASKIBA7 plus	*tig* gene (encodes TF) was amplified from pCold-TF (Takara) using primers X1946 (Forw XbaI with RBS) and X1947 (Rev HindIII) and inserted to the same sites of pASKIBA7plus.
tig(3A) (F44A/R45A/K46A)	P0A850	pLMB2079	pASKIBA7 plus	The 3 mutations (F44A/R45A/K46A) were introduced in pIMBB1605 using primer pair X2324-X2325
tig(4A) (M374A/Y378A/V384A/F387A)	P0A850	pIMBB1606	pASKIBA7 plus	*tig* 4A gene amplified from pLMB0038 [10] using primers X1946 and X1947 and was inserted to the XbaI-HindIII sites of pASKIBA7plus.
tig(4A,3A) (F44A/R45A/K46A/ M374A/Y378A/ V384A/F387A)	P0A850	pLMB2080	pASKIBA7 plus	The 3 mutations (F44A/R45A/K46A) were introduced in pIMBB1605 using primer pair X2324-X2325
secB	P0AG86	pIMBB351	pJW25	[11]
tig	P0A850	pCold-TF	pCold-TF	Takara.
Sec Translocase				
secYEG	P0AGA2 P0AG96 P0AG99	pIMBB336	pET610	A. Driessen, University of Groningen, Groningen, the Netherlands [12]
secA(1-901)	P10408	pIMBB10	pET5	[13]
secA(1-834)	P10408	pIMBB1296	pET5	SecA(noC-tail) [14]
secA(1-834)	P10408	pLMB2081	pBAD501	*secA*(noC-tail) gene amplified from pIMBB1296 using primers X157 and X1896 and was inserted to the NdeI-XhoI sites of pBAD501 [2]
secA(1-901)_ PatchA	P10408	pLMB1893	pET3a	SecA(noPatchA) The *secA* N1-314 PatchA/C98S DNA fragment was digested out from sgLMB0060 and inserted to pET3a *secA* 1-901 (pIMBB1280) after NdeI-MluI digestion.
His-*secA* (I304A/L306A)	P10408	pIMBB691	pET5	SecA(noSP) [14]

Appendix Table S3: Primers

X number	**DNA sequence** (5’-3’; Mutated codons are bold, restriction sites underlined)	**Description**
X157	GGCCCGTACATATGCTAATCAAATTGTTAAC	Forward *secA* primer introducing an NdeI site
X191	GGAATTCCATATGAAAAAGACAGCTATCGC	Forward primer for pro*ompA* amplification, introducing an NdeI site.
X193	CGGAATTCTTAAGCCTGCGGCTGAGTTAC	Reverse primer for pro*ompA* amplification, introducing an EcoRI site.
X558	GGGAATTCCATATGGCTCCGAAAGATAACACCT	Forward primer of *ompA* amplification. Anneals at Ala 22 introducing an NdeI 5’ end.
X1269	GACCCGCTCGAGAGCCTGCGGCTGAGTTACAACG	Reverse pro*ompA* primer introducing an XhoI site.
X1896	CCGGACCTCGAGTTATTGCAGGCGGCCATGGCACTGC	Reverse *secA* primer introducing an XhoI site
X1944	GCGGTCTAGATTAAGTATAA**GAAGGAG**ATATACATATGTCAGAACAAAACAACACTG	Forward *secB* primer introducing an rbs region and an XbaI site.
X1945	CCCAAGCTT**TTA**GGCATCCTGATGTTCTTCAGTACCTTC	Reverse *secB* primer introducing a stop codon and a HindIII site.
X1946	GCGGTCTAGATTAAGTATAA**GAAGGAG**ATATACATATGCAAGTTTCAGTTGAAACC	Forward *tig* primer introducing an rbs region and an XbaI site.
X1947	CCCAAGCTT**TTA**CGCCTGCTGGTTCATCAGCTCG	Reverse *tig* primer introducing a stop codon and a HindIII site.
X2324	GCGAAAAAAGTACGTATTGACGGC**GCGGCTGCT**GGCAAAGTGCCAATGAATATCGTT	Forward *tig* primer for generating F44A R45A K46A
X2325	AACGATATTCATTGGCACTTTGCC**AGCAGCCGC**GCCGTCAATACGTACTTTTTTCGC	Reverse *tig* primer for generating F44A R45A K46A

Appendix Table S4: Buffers

Buffer	**Composition**
A	50 mM Tris-HCl pH 8.0; 50 mM NaCl
B	6 M urea; 50 mM Tris-HCl pH 8.0; 50 mM NaCl
C	50 mM Tris-HCl pH 8.0; 50 mM NaCl; 1 mM DTT; 1 mM EDTA
D	50 mM Tris-HCl pH 8.0; 50 mM KCl; 5 mM $MgCl_2$
E	50 mM Tris-HCl; pH 8.0; 50 mM KCl; 5 mM $MgCl_2$; 5 µM $ZnCl_2$; 1mg/ml BSA; 1 mM DTT; 1 mM ATP
F	50 mM Tris-HCl pH 8.0; 50 mM KCl; 1 mM $MgCl_2$; 5 µM $ZnCl_2$
G	50 mM Ammonium Acetate pH 8
H	50 mM Tris HCl pH 7.5, 50 mM NaCl, and 1 mM TCEP
I	50 mM Tris-HCl pH 8.0; 0.5 M NaCl; 10% Glycerol v/v; 5 mM Imidazole; 2.5 mM PMSF; 50 µg/ml DNase I; 1 mM $MgCl_2$
J	50 mM Tris-HCl pH 8.0; 1 M NaCl; 10% Glycerol v/v; 5 mM Imidazole; 2.5 mM PMSF; 50 µg/ml DNase I; 1 mM $MgCl_2$
K	20 mM Tris-HCl pH 7.4; 50mM NaCl; 2.5 mM PMSF; 50 µg/ml DNase I; 1 mM $MgCl_2$
L	50 mM Tris-HCl pH 7.6; 200 mM KCl; 2.5 mM PMSF; 50 µg/ml DNase I; 1 mM $MgCl_2$
M	50 mM Tris-HCl pH 8.0; 20% Glycerol v/v; 10 mg/ml DNase I; 50 mg/ml RNase; 1 mM PMSF
N	50 mM Tris-HCl pH 8.0; 0.5 M NaCl; 10% Glycerol v/v; 8 M urea; 5 mM Imidazole
O	50 mM Tris-HCl pH 8.0; 0.5 M NaCl; 10% Glycerol v/v; 5 mM Imidazole

P	50 mM Tris-HCl pH 8.0; 0.5 M NaCl; 10% Glycerol v/v; 6 M Urea; 5 mM Imidazole
Q	50 mM Tris-HCl pH 8.0; 50 mM NaCl; 10% Glycerol v/v; 6 M Urea; 5 mM Imidazole
R	50 mM Tris-HCl pH 8.0; 50 mM NaCl; 10% Glycerol v/v; 6 M urea; 100 mM Imidazole
S	50 mM Tris-HCl pH 8.0; 50 mM NaCl; 6 M Urea; 10% Glycerol v/v
T	50 mM Tris-HCl pH 8.0; 1 M NaCl; 10% Glycerol v/v; 5 mM Imidazole
U	50 mM Tris-HCl pH 8.0; 50 mM NaCl; 10% Glycerol v/v; 5 mM Imidazole
V	50 mM Tris-HCl pH 8.0; 50 mM NaCl; 10% Glycerol v/v; 100 mM Imidazole
W	50 mM Tris-HCl pH 8.0; 50 mM NaCl; 10% Glycerol v/v
X	50 mM Tris-HCl pH 8.0; 50 mM NaCl; 50% Glycerol v/v
Y	20 mM Tris-HCl pH 7.4; 50 mM NaCl
Z	20 mM Tris-HCl pH 7.4; 100 mM NaCl
AA	20 mM Tris-HCl pH 7.4; 200 mM NaCl
AB	20 mM Tris-HCl pH 7.4; 350 mM NaCl
AC	50 mM Tris-HCl pH 8.0; 1 M NaCl
AD	50 mM Tris-HCl pH 7.6; 200 mM KCl
AE	50 mM Tris-HCl pH 7.6; 1.2 M KCl
AF	50 mM Tris-HCl pH 8.0
AG	50 mM Tris-HCl pH 8.0; 20% Glycerol v/v
AH	6 M Urea; 50 mM Tris-HCl pH 8.0
AI	50 mM Tris-HCl pH 8.0; 0.2 M Sucrose
AJ	50 mM Tris-HCl pH 8.0; 50 mM NaCl; 1 mM DTT; 1 mM EDTA; 6 M Urea
AK	0.2 M Sucrose; 1 mg/ml BSA in 50 mM Tris-HCl pH 8.0; 50 mM KCl; 1 mM $MgCl_2$; 5 μM $ZnCl_2$
AL	6 M Urea; 25 mM Tris pH 8.0; 25 mM KCl
AM	25 mM Tris pH 8.0; 25 mM KCl
AN	6 M Urea-d4 (98% atom D); 5 mM DTT; 95.5% (v/v) D_2O
AO	50 mM Tris-DCl corrected pD 8.0; 50 mM KCl; 1 mM $MgCl_2$; 4 μM $ZnSO_4$; 2 mM TCEP

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