Signal peptide hydrophobicity modulates interaction with the twin-arginine translocase

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Abstract

The general secretory pathway (Sec) and twin-arginine translocase (Tat) operate in parallel to export proteins across the cytoplasmic membrane of prokaryotes and the thylakoid membrane of plant chloroplasts. Substrates are targeted to their respective machineries by N-terminal signal peptides that share a common tripartite organization, however Tat signal peptides harbor a conserved and almost invariant arginine pair that are critical for efficient targeting to the Tat machinery. Tat signal peptides interact with a membrane-bound receptor complex comprised of TatB and TatC components, with TatC containing the twin-arginine recognition site. Here we isolated suppressors in the signal peptide of the Tat substrate, SufI, that restored Tat transport in the presence of inactivating substitutions in the TatC twin-arginine binding site. These suppressors increased signal peptide hydrophobicity, and co-purification experiments indicated that they restored binding to the variant TatBC complex. The hydrophobic suppressors could also act in *cis* to suppress substitutions at the signal peptide twin-arginine motif that normally prevent targeting to the Tat pathway. Highly hydrophobic variants of the SufI signal peptide containing four leucine substitutions retained the ability to interact with the Tat system. The hydrophobic signal peptides of two Sec substrates, DsbA and OmpA, containing twin lysine residues, were shown to mediate export by the Tat pathway and to co-purify with TatBC. These findings indicate that there is unprecedented overlap between Sec and Tat signal peptides and that neither the signal peptide twin-arginine motif nor the TatC twin-arginine recognition site are essential mechanistic features for operation of the Tat pathway.

Importance

Protein export is an essential process in all prokaryotes, The Sec and Tat export pathways operate in parallel, with the Sec machinery transporting unstructured precursors and the Tat pathway transporting folded proteins. Proteins are targeted to the Tat pathway by N-terminal signal peptides that contain an almost invariant twin-arginine motif. Here we make the surprising discovery that the twin-arginines are not essential for recognition of substrates by

the Tat machinery, and that this requirement can be bypassed by increasing the signal peptide hydrophobicity. We further show that signal peptides of *bona fide* Sec substrates can also mediate transport by the Tat pathway. Our findings suggest that key features of the Tat targeting mechanism have evolved to prevent mis-targeting of substrates to the Sec pathway rather than being a critical requirement for function of the Tat pathway.

Introduction.

The general secretory (Sec) and twin-arginine translocation (Tat) pathways operate in parallel to transport proteins across the cytoplasmic membranes of prokaryotes and the thylakoid membranes of plant chloroplasts. The Sec pathway translocates substrates in an unfolded conformation, whereas the Tat system transports folded proteins. Many Tat substrates contain redox cofactors that are non-covalently associated, and the Tat system is essential for photosynthesis and some modes of respiratory growth (reviewed in (1)).

Targeting of substrates to the Sec and Tat pathways is mediated by the presence of N-terminal signal peptides. Sec and Tat targeting sequences each have a recognisable tripartite structure with a positively-charged n-region, a hydrophobic h-region and a polar c-region that usually contains a cleavage site for leader peptidase (2, 3; Fig 1A). One of the primary differences between them is the presence of an almost invariant arginine pair in the n-region of Tat signal peptides. These consecutive arginines are reported to be mechanistically essential for substrate translocation by the Tat pathway, and even conservative substitutions to lysine are poorly tolerated (e.g. 4, 5). Twin arginines, however, are also compatible with the Sec pathway and some Sec signal peptides have paired arginines in their n-regions. A second key difference is the relative hydrophobicity of the two types of signal peptide. Tat targeting sequences are notably less hydrophobic than Sec signal peptides, and increasing the hydrophobicity of the TorA signal peptide re-routes a passenger protein from Tat to Sec (6, 7). Finally one or more positive charges is frequently found in the c-region of Tat signals that is not mechanistically required for Tat transport but serves to block interaction of the signal peptide with the Sec pathway (6-9). None-the-less, despite these differences, over half of the *E. coli* Tat signal peptides that were tested showed some level of engagement with the Sec pathway when fused to a Sec-compatible reporter protein (10).

Tat signal peptides interact with the membrane-bound Tat receptor complex. In *E. coli* the receptor contains TatA, TatB and TatC proteins, most likely in a 1:1:1 stoichiometry (11-13). The receptor is multivalent (14-16) and contains multiple copies of the TatABC heterotrimer (e.g. 17, 18). The primary recognition site for the Tat signal peptide is TatC (e.g.19-23), with

two conserved glutamates on the cytoplasmic face of TatC forming a patch that interacts with the signal peptide twin-arginines (24; Fig 1B). The signal peptide can also transition to a deep binding mode where it is inserted into the receptor complex, forming crosslinks to the transmembrane helix (TM) of TatB and TM5 of TatC (17, 19, 25, 26). Signal peptide insertion into the receptor drives structural reorganisation of the complex (13, 18, 26, 27) and the recruitment of further TatA molecules (19, 28-30). The assembled TatA oligomer mediates the transport of folded substrates across the membrane in an unknown manner, potentially by forming a translocation channel or by facilitating a localized weakening and transient disruption of the bilayer (31-33).

A recent study isolated genetic suppressors that restored transport activity to a Tat system that harbored an inactivating substitution in the TatC signal peptide binding site (27). These suppressing substitutions, located primarily in the TM of TatB, could also separately restore Tat transport to a substrate with a defective Tat signal peptide. Biochemical analysis revealed that these substitutions did not act to restore detectable signal peptide binding to the receptor complex but instead at least some of them induced conformational changes that apparently mimicked the substrate-activated state (27). In this work we have taken a complementary approach by searching for signal peptide suppressors able to restore Tat transport when the TatC signal peptide binding site was inactivated. We show that two separate inactive TatC variants, F94D and E103K, can be suppressed by single substitutions that increase the hydrophobicity of a Tat signal peptide. Remarkably, the same hydrophobic substitutions can suppress in *cis* by restoring Tat transport to a twin-arginine substituted signal peptide. Our results show that neither the twin-arginine motif nor its cognate recognition site on TatC are required for Tat transport activity. We further show that hydrophobic Sec signal peptides containing paired lysines can also mediate export by the Tat pathway pointing to an unexpected degree of overlap between Sec and Tat targeting requirements.

Results.

Isolation of suppressors of the inactivating TatC F94D substitution

A series of crosslinking studies, along with direct binding assays using purified TatC variants, have identified that the cytoplasmic N-terminal region and the cytoplasmic loop between TM2 and TM3 forms a binding site for the twin-arginine motif of Tat signal peptides (23, 24, 34, 35). Amino acid substitutions in the TM2-TM3 loop in particular are associated with loss of Tat activity, and residues F94 and E103 are almost completely invariant among TatC sequences from all three domains of life (34, 36, 37). Along with E15, E103 has been implicated in co-ordinating the positively charged twin-arginines of the signal peptide (24, 38; Fig 1B).

The twin-arginines are part of a larger consensus motif, S-R-R-x-F-L-K (Fig 1A) where the other amino acids are semi-conserved (2). The consensus phenylalanine is frequently present, particularly in bacterial Tat signal peptides, and for example is found in approximately 2/3^{rds} of *E. coli* Tat targeting sequences (39). It has been proposed through modelling studies that if the signal peptide n-region is in an extended conformation, TatC^{F94} may stack against this consensus F residue (40). We initially sought to test this hypothesis genetically.

It has been shown previously that a TatC^{F94D} substitution inactivates Tat transport and that strains harboring this substitution are unable to grow on media containing the detergent SDS (27; Fig 1D). This phenotype arises due to an inability to export two Tat substrates, AmiA and AmiC that remodel the cell wall during growth (41, 42). We used a fusion protein whereby the signal peptide of SufI, which has the consensus F residue (Fig 1A) was fused to the mature region of AmiA (27) and constructed a random library of codon substitutions at F8. We then screened this library against a strain lacking native *amiA*/*amiC* and harboring TatC^{F94D}, plating onto LB medium containing 2% SDS to select for suppressors of this inactivating substitution. However, after screening more than 1 000 clones we failed to isolate any suppressors of TatC^{F94D} from this library.

We therefore addressed whether it was possible to isolate substitutions elsewhere in the SufI signal peptide that would suppress TatC^{F94D}. To this end we constructed a random library of mutations throughout the SufI signal peptide coding region of the SufIss-AmiA fusion that had

some 13 000 members and an error rate of approximately 2%. After screening more than 20 000 individual transformants for the ability to grow in the presence of 2% SDS, we isolated 12 suppressors that supported growth on the detergent. Sequence analysis indicated that each of the suppressors shared a common substitution of serine at position 12 of the signal peptide to leucine (Fig 1C), and indeed this single S12L substitution was sufficient to support growth of a strain producing TatC^{F94D} on LB agar containing SDS (Fig 1D). Since the phenotypic growth test is largely qualitative, we also undertook a more quantitative assessment of growth of the strain co-producing TatC^{F94D} and SufI^{S12L}ss-AmiA by measuring growth curves in the presence of SDS. Fig 1E shows that the strain producing TatC^{F94D} and SufI^{S12L}ss-AmiA grew identically to the same strain producing wild type TatC and SufI^{S12L}ss-AmiA.

The SufI S12L substitution restore transport activity to a different substitution in the TatC signal peptide binding site

To determine whether the suppressor activity of the signal peptide S12L substitution was specific for TatC^{F94D} we tested whether this substitution could restore Tat transport to other TatC inactivating substitutions including P48L, V145E and Q215R located in consecutive periplasmic loops or E103K, located in the signal peptide binding site (37). Fig 2 shows that the inactivating TatC^{E103K} substitution could also be suppressed by the SufI^{S12L} variant, but transport activity was not restored to any of the substitutions in the periplasmic loops. We conclude that the S12L substitution specifically restores Tat transport to substitutions in the TatC signal peptide binding site.

The S12L substitution can restore transport activity to signal peptides that contain inactivating twin-arginine substitutions

Since the signal peptide S12L substitution can act in *trans* to suppress inactivating substitutions in the TatC signal peptide binding site, we next asked whether it could act in *cis* to rescue inactivating substitutions at the twin-arginine motif. Previously it has been shown that substitutions of one or both consensus arginines of the SufI signal peptide are poorly

tolerated (4), and indeed single substitutions of R6 to D, E, H, N or Q, or of R5R6 to KK, KH, KQ or HH in the SufIss-AmiA fusion are sufficient to prevent phenotypic growth of cells in the presence of SDS (27; Fig 3, Fig S1). Interestingly, however, introduction of the S12L substitution alongside any of the R6D, R6E, R6H, R6N, R6Q, or R5K/R6K restored strong growth of cells producing these fusion proteins in the presence of SDS (Fig 3A, Fig S1 panels B-G). The S12L substitution could also partially compensate for the R5K,R6Q substitution (Fig 3A, Fig S1 panel H), but could not rescue transport activity of the R5K,R6H or R5H,R6H variants (Fig 3B, Fig S1, panels I and J). For each of these variant signal peptides we confirmed that transport of the AmiA substrate remained strictly Tat-dependent since growth on SDS was not observed when the Tat system was absent (Fig S2). We conclude that the SufI^{S12L} signal peptide substitution can at least partially compensate for substitutions at the twin-arginine motif.

Single hydrophobic substitutions along the length the SufI signal peptide h-region can also suppress inactivating TatC substitutions in the signal peptide binding site

The h-regions of Tat signal peptides are less hydrophobic than Sec signal sequences, containing significantly more glycine and less leucine residues (6). The S12L substitution replaces a polar residue near the start of the SufI signal peptide h-region with a highly hydrophobic amino acid, markedly increasing its hydrophobicity score (Table 1). To test whether single hydrophobic substitutions elsewhere in the SufI signal peptide h-region could also suppress Tat transport defects, we increased hydrophobicity of the h-region by constructing individual A11L, G13L, A15L, A18L, G19L and A20L variants. Fig 4A shows that when each of these individual substitutions was introduced into the SufIss-AmiA construct and produced in a strain harboring *tatC*^{F94D}, phenotypic growth on SDS was restored. When this was examined semi-quantitatively by following growth curves (Fig 4B), it could be seen that the G13L, A15L and G19L substitutions suppressed the *tatC*^{F94D} allele more strongly than A11L, A18L or A20L. Table 1 shows that the substitutions that give the biggest increase in hydrophobicity result in the strongest level of suppression. It should be noted that the

189 SufIss^{G13L} substitution appeared to result in a very low level of the fusion protein being routed
190 to the Sec pathway as weak growth could be detected in a strain lacking the Tat pathway (Fig
191 4A, Fig 4C). None of the other substitutions, however, led to any detectable transport by Sec.
192
193 We next tested whether these further hydrophobic substitutions could also suppress a second
194 signal peptide binding site substitution, TatC^{E103K}. It can be seen (Fig S3A, Fig 4D) that these
195 variants could also compensate for loss of Tat activity resulting from this substitution. They
196 could not, however, compensate for any of TatC P48L, V145E and Q215R (not shown). As
197 seen for the suppression of TatC^{F94D}, the substitutions giving the biggest increase in
198 hydrophobicity (S12L, G13L and G19L) restored the highest level of Tat activity in the
199 presence of TatC^{E103K} (Fig 4D). Finally, we also tested whether the strongest suppressors of
200 TatC^{E103K} could also rescue transport in *cis* when introduced into the twin lysine variant of the
201 SufI signal peptide. Fig S3B indicates that similar to the S12L substitution, introduction of any
202 of the G13L, A15L and G19L substitutions into KK-SufIss-AmiA restored strong growth of cells
203 producing these fusion proteins in the presence of SDS. We conclude that increasing h-region
204 hydrophobicity can suppress transport defects associated with either the signal peptide twin-
205 arginine motif or the signal peptide binding site.
206
207 **The h-region suppressors support transport of full-length SufI**
208 To assess the level of Tat transport mediated by these hydrophobic variants of the SufI signal
209 sequence, we introduced the S12L, G13L, A15L and G19L substitutions individually into a
210 construct encoding C-terminally His-tagged but otherwise wild-type SufI. We initially
211 expressed these in a strain producing native TatABC and fractionated cells to obtain the
212 periplasm. Fig 5A shows that each of these single hydrophobic variants of the SufI signal
213 peptide supported strong export of SufI-His. Transport of these SufI-His variants was strictly
214 dependent on the Tat pathway since no periplasmic SufI-His could be detected when the Tat
215 pathway was absent (Fig S4A).

Next we assessed the degree of transport mediated by these variant signal peptides in cells producing TatABC^{F94D}. It can be seen (Fig 5B) that although wild type SufI-His was not exported in the presence of TatC^{F94D}, transport was detected when any of the single hydrophobic substitutions were present in the signal peptide. The S12L substitution in particular could strongly suppress TatC^{F94D}, with high levels of SufI-His detected in the periplasm when the signal peptide harbored this mutation. These same signal peptide substitutions could also restore good transport of SufI-His in the presence of the inactivating TatC^{E103K} substitution (Fig S4B).

Since the hydrophobic substitutions can act in *cis* to restore transport activity to a twin lysine variant of the SufI signal peptide twin-arginine motif, we assessed the export of the KK variant of SufI-His harboring the S12L substitution. Fig 5C shows that there was clear Tat transport activity conferred on the twin lysine signal peptide variant by the presence of the S12L suppressor. Taken together the results presented so far indicate that the signal peptide consecutive arginines and the TatC twin-arginine recognition site are not essential mechanistic features for operation of the Tat pathway and substitutions in either of these can be at least partially compensated for by an increase in signal peptide hydrophobicity.

The h-region suppressors restore signal peptide binding to TatBC

A previous study identified suppressors in the TatB component that could also restore transport activity to substitutions in the TatC twin-arginine binding site. It was shown that substrate precursors could be co-purified with wild type TatBC complexes but did not co-purify when the signal peptide binding site was mutated, even in the presence of the TatB suppressors. Thus it was concluded that the TatB suppressors did not detectably restore binding of signal peptides to the TatBC complex (27). To determine whether the suppressors we identified here that increase signal peptide hydrophobicity could restore binding to TatBC complexes harboring the TatC^{F94D} substitution, we co-produced FLAG-tagged variants of SufI with these suppressors alongside TatB and His-tagged TatC. After purification of TatBC complexes from digitonin-treated cell lysates, we assessed the level of co-purifying SufI by

immunoblotting. As shown in Fig 6, the single substitutions S12L, G13L, A15L or G19L in the SufI signal peptide did not detectably affect interaction of SufI with wild type TatBC, since qualitatively similar levels of FLAG-tagged SufI were seen to co-purify with TatBC-His. When the F94D substitution was present in His-tagged TatC, no SufI-FLAG was co-purified with the variant TatBC-His complex (Fig 6A), even though SufI was clearly detected in the cell lysate (Fig S5). However, when the S12L, G13L, A15L or G19L substitutions were introduced into SufI, it could now be detected in the fractions containing purified TatBC^{F94D}-His. These observations indicate that the SufI h-region suppressors restore some degree of signal peptide binding to the TatBC^{F94D} complex.

Highly hydrophobic signal peptides are compatible with the Tat pathway

It has previously been reported that the relatively low hydrophobicity of Tat signal peptides partially prevents their routing to the Sec pathway (6). It is not clear, however, whether low h-region hydrophobicity is a mechanistic requirement for engagement with Tat. To explore this in more detail, we investigated the effect of further increasing the hydrophobicity of the SufI signal peptide on transport of the SufIss-AmiA fusion. To this end we introduced a S12L/G13L double substitution, and two quadruple substitutions, S12L/G13L/I14L/A15L and C17L/A18LG19L/A20L, into the SufI signal sequence. These substitutions markedly increase the signal sequence hydrophobicity score, bringing it into the range of the Sec signal sequences of OmpA and DsbA (Table 1).

Fig 7A shows that each of these SufIss-AmiA fusion proteins was able to support growth on solid medium in the presence of SDS, although growth was also seen in a strain lacking the Tat pathway, indicating that there is some export of these more hydrophobic SufIss-AmiA fusion proteins by Sec. These findings were confirmed by monitoring growth of these strains in liquid culture (Fig 7B,C). However it is clear that in the absence of a functional Tat system, growth in the presence of SDS was much poorer than when the Tat system was present. This observation suggests that there must be some recognition of these hydrophobic signal peptide variants by the Tat pathway. To confirm this, we co-produced FLAG-tagged

S12L/G13L/I14L/A15L and C17L/A18LG19L/A20L SufI variants alongside TatBC-His. When His-tagged TatC was purified from digitonin-solubilized cell lysates, each of these hydrophobic SufI-FLAG variants was co-purified (Fig 6A), indicating that they retained the ability to interact with the TatBC complex. Taken together, these results show that highly hydrophobic signal peptides are mechanistically compatible with the Tat pathway.

The OmpA and DsbA signal peptides functionally interact with the Tat pathway

Our results collectively show that the hallmark twin-arginines of Tat signal peptides are not a mechanistic requirement for Tat-dependent transport and that a single arginine or twin lysines in the n-region are compatible with the Tat pathway if compensatory mutations are introduced that increase the hydrophobicity of the signal peptide. Interestingly, many Sec-dependent signal peptides share these parameters (Table S1), raising the possibility that *bona fide* Sec signal peptides may be able to interact with the Tat pathway. To explore this we selected two well-studied Sec signal peptides – those of OmpA, which is a post-translational Sec substrate and of DsbA which directs co-translational translocation (43, 44; Table 1) - and fused their signal peptides to the mature portion of AmiA. We also made two additional constructs where we introduced a 'Sec-avoidance' lysine residue into the signal peptide c-regions, to reduce interaction with the Sec pathway (8).

Fig 8A shows that there is Sec-dependent transport of AmiA mediated by the OmpA signal peptide as there is strong growth of the Δ*tat* strain producing OmpAss-AmiA in the presence of SDS. Introduction of a lysine at position 18 of the OmpA signal peptide clearly reduces interaction with the Sec pathway as growth of the Δ*tat* strain producing this variant is significantly reduced. However, there is good growth of *tat*$^+$ strain producing this variant fusion protein, indicating that some of this fusion must be interacting with the Tat pathway. Similarly, Fig 8B shows that there is some low level growth of the Δ*tat* strain producing DsbAss-AmiA in SDS-containing medium, which is reduced by inclusion of a Sec-avoidance lysine in the c-region of the DsbA signal peptide. By contrast, the *tat*$^+$ strain harboring either of these fusion

proteins shows markedly stronger growth in the presence of SDS, indicating that the DsbA signal peptide is productively engaging with the Tat machinery.

To confirm that these signal peptides are able to interact with Tat, we co-produced C-terminally FLAG-tagged variants of full length OmpA or full length DsbA alongside TatBC-His. When His-tagged TatC was purified from digitonin-solubilized cell lysates, DsbA-FLAG, which migrated very close to the expected mass of 24.1KDa, was seen to co-purify (Fig 8C). This co-purification was clearly dependent on the presence of the Tat proteins since when membranes were removed by an ultra-centrifugation step, the cytoplasmic form of DsbA-FLAG was no longer isolated by Ni-affinity purification (Fig 8D). We conclude that FLAG-tagged, but otherwise native DsbA can interact with TatBC. By contrast we were not able to detect co-purification of FLAG-tagged OmpA with TatBC under these conditions (Fig 8C). However in these experiments we noted that OmpA-FLAG migrated at a lower mass than the predicted size of the tagged protein (38.2kDa), or of folded OmpA (which migrates at an estimated mass of approximately 30kDa; (45)), raising the possibility that it may have been subjected to proteolysis. We therefore took a second approach to assessing whether the OmpA signal peptide could interact with TatBC by fusing the OmpA signal peptide variant containing the K18 insertion to the N-terminus of mature SufI, and co-producing it with TatBC-His. Fig 6C indicates that this fusion protein could indeed be co-purified alongside TatB and His-tagged TatC indicating that the OmpA signal peptide is able to interact with the TatBC complex.

Discussion

In this study we have sought to identify SufI signal sequence variants that restore Tat transport activity in the presence of substitutions that inactivate the twin-arginine recognition site on TatC. Our results have shown that an increase in signal peptide hydrophobicity can overcome two different inactivating substitutions, $TatC^{F94D}$ and $TatC^{E103K}$, and that these suppressors act to restore detectable binding of the SufI signal sequence to the variant $TatBC^{F94D}$ complex. We further showed that the same hydrophobic substitutions can act in *cis* to compensate for a range of inactivating substitutions at the SufI signal peptide twin-arginine motif. These results demonstrate that neither the consecutive arginines of the signal peptide nor the conserved recognition site on the cytoplasmic surface of TatC are mechanistically essential for operation of the Tat pathway, and that they can be bypassed if the signal peptide hydrophobicity is increased. Taken together our findings indicate that the signal peptide features that can facilitate interaction with the Tat pathway are remarkably similar to those that facilitate interaction with Sec, namely the presence of at least one basic charge in the n-region, and a relatively hydrophobic h-region. Indeed we show that even a highly hydrophobic signal peptide that naturally directs its passenger into the co-translocational Sec pathway can functionally engage with the Tat system.

If the Tat pathway can interact with hydrophobic signal peptides lacking the twin-arginine motif, why then do almost all Tat substrates that have been identified contain paired arginine residues and only moderately hydrophobic h-regions? In prokaryotes and plant chloroplasts, the Tat system always co-exists with the Sec pathway. In bacteria, ribosomal-associated signal recognition particle (SRP) and cytosolic or ribosomal-bound SecA capture Sec substrates at an early stage of biogenesis, at least partially through interaction with their signal sequences (46, 47). Signal sequence hydrophobicity is key sorting feature for Sec substrates; highly hydrophobic signals generally interact with SRP whereas those with lower hydrophobicity bind to SecA (46). Photocrosslinking and/or genetic studies have indicated that Tat signal peptides interact with ribosomally-bound trigger factor and with general cytoplasmic

chaperones including DnaK (48-51), but no crosslinks to SecA have been reported, and *in vitro* analysis indicates that Tat signal peptides do not productively engage with SecA to the same extent as a Sec signal peptide (52). It is therefore likely that Tat signal peptides evolved lower hydrophobicity to avoid the targeting pathways that feed into the Sec translocon, and that the paired arginines and the twin-arginine binding site are necessary features to strengthen recognition of these weakly hydrophobic signal peptides by the Tat machinery. In this context it is interesting to note that although paired arginines in the signal peptide n-region are compatible with the Sec pathway, this pairing is relatively rare in Sec signal peptides, at least in *E. coli*, being found in only five of the 244 probable Sec signal peptides listed in Table S1 (compared with 53 that have paired lysines). If lysine and arginine are equivalent in the amino terminal region of a Sec signal peptide, as implied by kinetic analysis (53), this might suggest that there is selection pressure against the presence of paired arginines in Sec signals.

The presence of one or more positively charged amino acids in the c-region of Tat signal peptides is a further feature that has no mechanistic requirement for Tat translocation but leads to rejection of these signal sequences by the Sec pathway (6, 8, 9). C-terminal positive charges may act at a late stage during Sec translocation when the signal peptide is already engaged with the Sec translocon, and Sec avoidance motifs are particularly abundant in membrane proteins that require the dual action of the Sec and Tat pathways for their assembly (7, 54). Here the Tat-dependent signal sequence (which is internal to the protein and follows a series of Sec-dependent transmembrane domains) has several c-region positive charges that result in abortive interaction with the Sec pathway, freeing up the sequence to be recognized by Tat (7). Taken together it is clear that there is strong selective pressure, particularly at the level of Tat signal peptides, to refine features that minimise mis-targeting to the Sec pathway.

Our findings show that signal peptides with either twin lysines or an unpaired arginine, coupled with a moderately hydrophobic h-region can functionally interact with the Tat pathway.

372 Inspection of all of the signal peptides present at the N-termini of *E. coli* MG1655 proteins
373 identified using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP; 55) indicates that some
374 44% of Sec signal peptides contain either KR, RK, KK, RD, RE, RH, RN, RQ adjacent to their
375 h-regions (Table S1) and therefore potentially have the capability of engaging with the Tat
376 pathway. Whether any of these would ever target to the Tat pathway *in vivo* is not clear, since
377 presumably under standard conditions *E. coli* synthesizes sufficient targeting factors to ensure
378 that Sec substrates are efficiently channelled into the Sec pathway. However, it should be
379 noted that in *Bacillus subtilis*, hyper-production of a normally Sec-dependent lipase results in
380 overflow into the Tat pathway (56), raising the possibility that transient re-routing of substrates
381 to the Tat pathway may occur on occasions where cells undergo secretion stress.

Materials and Methods

Strain and plasmid construction.

Strains used in this study are MC4100 derivatives (57). Strain MC4100 Δ*amiA* Δ*amiC* Δ*tatABC* (F-Δ*lacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301* Δ*amiA* Δ*amiC* Δ*tatABC*) was used for signal peptide library screening and for SDS growth tests with signal peptide-AmiA fusion proteins (27). Strain DADE (as MC4100, Δ*tatABCD* Δ*tatE*; 58) was used for SufI transport assays and DADE-P (as DADE, *pcnB1 zad*-981::Tn*10*d (Kan^r; 59) was used for co-purification experiments.

All plasmids used and constructed in this study are given in Table S2. Point mutations in plasmids were introduced by Quickchange site-directed mutagenesis (Stratagene) using the primers listed in Table S3. Plasmid pTAT101 was used for low-level production of TatA, TatB and TatC (37). Plasmid pSUSufIss-mAmiA was used to produce SufIss-AmiA, where the SufI signal peptide is fused to the mature portion of AmiA (27). Plasmid pQE80-SufIhis was used to produce his-tagged SufI (27).

Plasmids pSUDsbAss-mAmiA, pSUDsbAssi16K-mAmiA (with an additional lysine codon inserted after codon 16 of the DbsA signal peptide), pSUOmpAss-mAmiA, and pSUOmpAssi18K-mAmiA (with an additional lysine codon inserted after codon 18 of the OmpA signal peptide) were constructed according to (27). Briefly, DNA fragments encoding DsbAss, DsbAssi16K, OmpAss, and OmpAssi18K were amplified by PCR using MC4100 genomic DNA as template, using primer pairs DsbAss-FE / DsbAss-R, DsbAss-FE / DsbAss16inK-R, OmpA-FE / OmpAss-R, and OmpA-FE / OmpA18inK-R, respectively. DNA fragments encoding the corresponding mature domain of AmiA were amplified by PCR using MC4100 genomic DNA as template with primer pairs OmpA-mAmiA-F / amiA-mRX, or DsbA-mAmiA-F / amiA-mRX. The DNA fragments encoding the signal peptides and the mature domain of AmiA were fused by overlap extension PCR, giving DNA fragments DsbAss-mAmiA, DsbAssi16K-mAmiA, OmpAss-mAmiA, and OmpAi18Kss-mAmiA, which were finally cloned into pSU18 vector following digestion with *Eco*RI and *Xba*I.

Plasmid pFAT75BC-SufIFLAG was modified from pFAT75ΔA-SufIhis (18) via quickchange using primers FAT75SufIFLAG-1 / FAT75SufIFLAG-2. Plasmid pFATBChis-SufIFLAG was modified from pFATSufIFLAG via quickchange using primers FAT75TatChis-1 / FAT75TatChis-2. Plasmid pFATBChis-OmpAssi18KSufIFLAG has the SufI signal peptide coding region substituted for DNA encoding OmpAssi18K, and was constructed using a restriction enzyme-free cloning method according to (60). Briefly a DNA fragment covering OmpAssi18K was PCR amplified using pSUOmpAssi18K-mAmiA as template, with primer pair FATHF-OmpA-F / FATHF-OmpA18K-R. The resultant DNA fragment was used as a primer to amplify the whole pFATBChis-SufIFLAG plasmid using the PCR program: 95°C 2min followed by 15 cycles of 95 °C 30s, 48 °C 1.5 min and 68 °C 15 min and a final extension at 68 °C for 10 min. The PCR product was subject to *Dpn*I digestion and introduced into *E. coli* JM109 competent cells by transformation. The resultant plasmid was verified by DNA sequencing. Plasmids pQEBChis-OmpAFLAG and pQEBChis-DsbAFLAG were used for co-production of TatB, his-tagged TatC and FLAG-tagged OmpA or DsbA, respectively, and were constructed as follows. A DNA fragment encoding TatB and his-tagged TatC was amplified using pFATBChis-SufIFLAG as template with primer pair QEF / FAT75TatChis-2, and was ligated, via an *Apa*I restriction site, to a DNA fragment encoding FLAG-tagged OmpA (that was amplified using MC4100 genomic DNA as template with primer pair FATHF-OmpA-F / OmpAFLAG-SR) or FLAG-tagged DsbA (amplified similarly using primer pair FATHF-OmpA-F / DsbAFLAG-SR). The ligated fragment was enriched by using the ligation mixture as a template in a PCR reaction with primer pair QEF / OmpAFLAG-SR, or QEF / DsbAAFLAG-SR, presectively. The amplified fragment was gel-purified, digested with *Eco*RI and *Sal*I and cloned into similarly-digested pQE80. Constructs were verified by DNA sequencing.

Mutant library construction and screening.

To construct a random library of substitutions at codon 8 of the SufI signal peptide, site-directed mutagenesis was carried out via Quickchange using a pair of random primers SufIF8X1 and SufIF8X2 (Table S3) and pSUSufIss-mAmiA as template. The PCR product

437 was subsequently introduced into XL1-Gold ultra-competent cells (Agilent). Transformants
438 were scraped from plates, resuspended in LB, pooled and cultured overnight after which
439 plasmid DNA was isolated and taken as the F8X random library. The library contained
440 approximately 5 000 clones and random sequencing of eight of them revealed substitutions of
441 the TTC codon to CTT, ATC GGT, GTG, GGT, CAA, AGA and GGG.

442 The signal peptide mutagenesis library in plasmid pSUSufIss-mAmiA was constructed as
443 described (27). Briefly, an error-containing DNA fragment covering the *sufI* signal sequence
444 was amplified by error-prone PCR using primers SufIF and SufIR and pSUSufIss-mAmiA as
445 template. This fragment was used as a megaprimer to amplify the whole pSUSufIss-mAmiA
446 plasmid. The amplified plasmid was introduced into XL1-gold ultra-competent cells following
447 nick repair using T4 polynucleotide kinase and T4 ligase. Transformants were scraped from
448 plates, resuspended in LB, pooled and used to inoculate fresh LB to an initial OD_{600} of 0.2.
449 Cells were grown at 37 °C until OD_{600} reached 2 after which plasmid DNA was prepared and
450 taken as the signal peptide mutagenesis library.
451 For library screening, plasmid pTAT101 harboring the *tatC* point substitution of interest (along
452 with wild type *tatAB*) was introduced into MC4100 Δ*amiA* Δ*amiC* Δ*tatABC*. Subsequently the
453 mutant library was introduced and cells were plated onto LB agar containing 2% SDS.
454 Plasmids were isolated from colonies growing on this selective medium and mutations
455 identified by sequencing.
456
457 **Protein methods.**
458 Co-purification of TatBC-substrate complexes was carried out as described (27). Briefly, an
459 overnight culture of DADE-P harboring plasmid pFATBChis-SufIFLAG or its derivatives was
460 subcultured in LB supplemented with 0.5% glycerol and appropriate antibiotics for 2.5 hours
461 at 37 °C with shaking. Following supplemented with 0.4 mM isopropyl-β-D-galactopyranoside
462 (IPTG), cells were incubated overnight at 30 °C. The following morning, cells were harvested,
463 resuspended in 200μL of 2X lysis buffer (100 mM NaH_2PO_4 pH 8.0, 600 mM NaCl, 40 mM

imidazole, 50 mg lysozyme, DNase I, and protease inhibitor), and mixed gently at room temperature for one hour. Cells were then frozen at -80 °C for 1 hour and thawed at room temperature. An equal volume of 2.5% digitonin was added to the cells and the samples were solubilized for 1 hour at 4°C. The insoluble material was pelleted by centrifugation at 4 °C. A 30 μL of the supernatant was mixed with 2x Laemmli buffer which was taken as the input sample and the remaining supernatant was mixed with 50 μL wash buffer-equilibrated nickel beads (Profinity™ IMAC Ni-Charged Resin, Bio-Rad, catalog number 156-0131) for one hour. The nickel beads were pelleted, washed three times with 1 mL wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 40 mM imidazole, 0.03 % digitonin) and then mixed with 100 μL elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 700 mM imidazole, 0.03 % digitonin). The beads were incubated for 10 min with shaking and then pelleted. The supernatant (elution fraction) was taken, mixed with an equal volume of 2x Laemmli buffer, and 20 μl of the sample was subjected to SDS-PAGE followed by western-blotting with anti-His (Anti-6X His tag® antibody [GT359] (HRP), Abcam, catalog number ab184607), anti-TatB (61), or anti-FLAG antibodies (Monoclonal ANTI-FLAG® M2 antibody produced in mouse. Sigma catalog number F1804). Secondary antibody was goat anti-rabbit IgG (HRP Conjugate, Bio-Rad, catalog number 170-6515) or goat anti-mouse IgG (HRP Conjugate, Bio-Rad, catalog number 1706516).

Subcellular fractionation was carried out as described previously (27). Briefly, overnight cultures of strain DADE harboring pTAT101 or the cognate empty vector pTH19kr along with pQE80-SufIhis or its derivatives were subcultured at 1:50 in LB supplemented with 1mM IPTG and grown at 37 °C until OD_{600} reached 1. Where the RR-KK substitution was present in the SufI, no IPTG was used (as for unknown reasons this substitution results in high level expression of SufI even in the absence of IPTG). For whole cell samples, cells were pelleted from 5 mL of the culture, resuspended in 250 μL resuspension buffer (50mM Tris-HCl, pH 7.6, 2 mM EDTA), and lysed by sonicating for 15 s. The cell lysate was mixed with an equal volume of 2x Laemmli buffer and boiled at 95 °C for 10 min. For preparation of periplasm, cells were

491 pelleted from 20 mL of the culture and resuspended in 500 μL fractionation buffer (20mM Tris-
492 HCl, pH 7.6, 2 mM EDTA, 20% sucrose (w/v)). 0.6 mg/mL freshly made lysozyme was added
493 and the cells were incubated at room temperature for 20 min. The cells were then pelleted by
494 centrifugation and the supernatant was taken and mixed with equal volume of 2x Laemmli
495 buffer. Aliquots (20 μL) of the whole cell or periplasmic fraction samples were separated by
496 SDS-PAGE (10% acrylamide) followed by western blotting with anti-His, or anti-his and anti-
497 RNA polymerase β-subunit mixed antibodies.
498
499 **Prediction of Sec signal peptides.**
500 All of the protein sequences encoded by *E. coli* MG1655 were analyzed using the SignalP 4.1
501 server (http://www.cbs.dtu.dk/services/SignalP; (55)) with parameters 'Gram-negative
502 bacteria' and 'input sequence do not include TM regions' selected. Inner membrane proteins
503 were removed manually from the output Sec substrate candidates.

Acknowledgements

This work was supported by the China Scholarship Council (through a studentship to QH). TP is a Wellcome Trust Investigator.

Signal peptide	Sequence	Hydrophobicity
WT SufI	MSLSRRQFIQASGIALCAGAVPLKASA	1.75
SufI 11L	MSLSRRQFIQLSGIALCAGAVPLKASA	1.91
SufI 12L	MSLSRRQFIQALGIALCAGAVPLKASA	2.11
SufI 13L	MSLSRRQFIQASLIALCAGAVPLKASA	2.08
SufI 15L	MSLSRRQFIQASGILLCAGAVPLKASA	1.92
SufI 18L	MSLSRRQFIQASGIALCLGAVPLKASA	1.91
SufI 19L	MSLSRRQFIQASGIALCALAVPLKASA	2.08
SufI 20L	MSLSRRQFIQASGIALCAGLVPLKASA	1.91
SufI 12L,13L	MSLSRRQFIQALLIALCAGAVPLKASA	2.43
SufI 12L,13L,14L,15L	MSLSRRQFIQALLLLLCAGAVPLKASA	2.53
SufI 17L,18L,19L,20L	MSLSRRQFIQASGIALLLLLVPLKASA	2.48
WT OmpA	MKKTAIAIAVALAGFATVAQA	2.52
OmpA i18K	MKKTAIAIAVALAGFATKVAQA	2.52
WT DsbA	MKKIWLALAGLVLAFSASA	2.57
DsbA i16K	MKKIWLALAGLVLAFKSASA	2.57

Table 1. Relative hydrophobicities of signal peptide variants used in this work. In each case the h-region sequence used to calculate the score is shown underlined. Hydrophobicity was scored using grand average of hydropathy (GRAVY) value at http://www.gravy-calculator.de/.

511 **Figure Legends**

512

513 **Figure 1.** A. Schematic representation of Sec and Tat signal peptides. The sequence of the
514 OmpA and DsbA Sec-targeting signals and the SufI Tat-targeting signal peptide are shown.
515 Positive charges in the signal peptide n-regions are shown in underline, and the amino acids
516 of the SufI Tat consensus motif are shown in red. B. Models of *E. coli* TatC (*left*) side view,
517 with F^{94} and E^{103} residues that are located in the signal peptide binding site given in pink and
518 red, respectively, and (*right*) view of the cytoplasmic face, with E^{15} additionally shown. C.
519 Alignment of the amino acid sequence of twelve suppressors in the SufI signal peptide that
520 compensate for the TatC F94D substitution. D and E. Cells of strain MC4100 Δ*amiA* Δ*amiC*
521 Δ*tatABC* harboring pTH19kr (empty vector; annotated Δ*tat*) or pTAT101 producing wild type
522 TatAB along with either wild type TatC (*tat*$^+$) or $TatC^{F94D}$ (*tatABC*^{F94D}) and a compatible plasmid
523 encoding either pSUSufIss-mAmiA or pSUSufI^{S12L}ss-mAmiA, as indicated, were sub-cultured
524 at 1:100 into fresh LB medium following overnight growth and: D. incubated for 3 hours at 37
525 °C with shaking. Cells were pelleted, re-suspended in sterile PBS supplemented with
526 appropriated antibiotics to an OD_{600} of 0.1 and 8 μL of sample was spotted onto LB agar or
527 LB agar containing 2% SDS Plates were incubated at 37 °C for 16 hours, or E. supplemented
528 with 0.5% SDS (final concentration) and grown at 37 °C without shaking. The optical density
529 at 600nm was monitored every 20min using a plate reader. Error bars are ± SD, *n*=3 biological
530 replicates.

531

532 **Figure 2.** The SufI^{S12L} substitution can restore Tat transport to $TatC^{E103K}$. A and B. Overnight
533 cultures of strain MC4100 Δ*amiA* Δ*amiC* Δ*tatABC* harboring either pSUSufIss-mAmiA or
534 pSUSufI^{S12L}ss-mAmiA alongside plasmid pTAT101 producing wild type TatAB along with the
535 indicated substitution of TatC, as indicated, were sub-cultured at 1:100 dilution and: A. grown
536 for a further 3 hours at 37 °C, pelleted, re-suspended to an OD_{600} of 0.1 and 8 μL of sample
537 was spotted onto LB agar or LB agar containing 2% SDS. Plates were incubated at 37 °C for

538 16 hours, or B. supplemented with 0.5% SDS (final concentration) and grown at 37 °C without
539 shaking. The optical density at 600nm was monitored every 20min using a plate reader. Error
540 bars are ± SD, *n*=3 biological replicates.

541 **Figure 3.** The SufI^{S12L} substitution can act in *cis* to suppress inactivating substitutions in the
542 SufI signal peptide twin-arginine motif. A and B. Overnight cultures of strain MC4100 Δ*amiA*
543 Δ*amiC* Δ*tatABC* harboring either pTH19kr alongside pSUSufIss-mAmiA (Δ*tat*) or pTAT101
544 (producing wild type TatABC) alongside either unsubstituted pSUSufIss-mAmiA (*tat*$^+$) or
545 pSUSufIss-mAmiA encoding the indicated substitutions in the SufI signal peptide were sub-
546 cultured at 1:100 dilution and grown for a further 3 hours at 37 °C, pelleted, re-suspended to
547 an OD_{600} of 0.1 and 8 μL of sample was spotted onto LB agar or LB agar containing 2% SDS.
548 Plates were incubated at 37 °C for 16 hours.
549
550 **Figure 4.** Single leucine substitutions throughout the SufI signal peptide h-region can
551 suppress the TatC F94D and E103K substitutions. A - C. Overnight cultures of strain MC4100
552 Δ*amiA* Δ*amiC* Δ*tatABC* harboring either pTH19kr (Δ*tat*) or pTAT101 producing TatABC^{F94D}
553 along with pSUSufIss-mAmiA producing the indicated substitution in the SufI signal peptide
554 were sub-cultured at 1:100 dilution and: A. grown for a further 3 hours at 37 °C, pelleted, re-
555 suspended to an OD_{600} of 0.1 and 8 μL of sample was spotted onto LB agar or LB agar
556 containing 2% SDS. Plates were incubated at 37 °C for 16 hours, or B. and C. supplemented
557 with 0.5% SDS (final concentration) and grown at 37 °C without shaking. D. MC4100 Δ*amiA*
558 Δ*amiC* Δ*tatABC* harboring pTAT101 producing TatABC^{E103K} along with pSUSufIss-mAmiA
559 producing the indicated substitution in the SufI signal peptide was subcultured into LB
560 containing 0.5% SDS and grown at 37 °C without shaking. For all growth curves the optical
561 density at 600nm was monitored every 20min using a plate reader. Error bars are ± SD, *n*=3
562 biological replicates.
563

564 **Figure 5.** Analysis of SufI export mediated by signal peptide leucine substitutions in the
565 TatC^{F94D} background or when combined with a signal peptide twin-lysine substitution. A. and
566 B. *E. coli* strain DADE co-producing his-tagged but otherwise native SufI, or SufI with the
567 indicated single leucine substitutions in the signal peptide (from a pQE80 plasmid) alongside
568 either A. wild-type TatABC or B. wild type TatAB and TatC^{F94D} (from pTAT101). Strain DADE
569 co-producing his-tagged but otherwise native SufI alongside an empty vector was used as a
570 negative control (lanes annotated 'Δ*tat* WT SufI'), or C. Strain DADE producing his-tagged
571 SufI harboring an R5K,R6K double substitution (SufI KK) and with an additional S12L
572 substitution where indicated, alongside either empty vector pTH19kr (Δ*tat*) or pTAT101
573 encoding wild type TatABC (*tat*$^+$). In each case strains were grown to mid-log phase and
574 fractionated into whole cell (upper panels) and periplasm (lower panels), then analyzed by
575 Western blot with anti-6X His tag® or anti-RNA polymerase β subunit antibodies (cytoplasmic
576 control protein). wc – whole cell. Equivalent volumes of sample were loaded for each of the
577 whole cell samples, and for each of the periplasmic samples.

578

579 **Figure 6.** Hydrophobic variants of the SufI signal peptide mediate binding to TatBC and
580 TatBC^{F94D}. A. Cells of strain DADE-P co-producing C-terminally FLAG-tagged SufI with its
581 native signal peptide (WT SufI) or harboring the indicated leucine substitutions, alongside TatB
582 and C-terminally His-tagged TatC or TatC^{F94D} were lysed and incubated with digitonin and His-
583 tagged TatC was isolated with Ni-charged beads. Following elution of bound TatC-His,
584 equivalent volumes of the eluate from each sample were analyzed by western blotting with
585 anti-His, anti-TatB, or anti-FLAG antibodies as indicated. DADE-P co-producing C-terminally
586 FLAG-tagged SufI with its native signal peptide alongside TatB and non-tagged TatC (lane
587 annotated 'No Histag) was used as a negative control. Equivalent volumes of sample were
588 loaded in each lane. B. Cells of DADE-P co-producing C-terminally FLAG-tagged SufI fused
589 to the OmpA signal peptide harboring a lysine insertion at codon 18, alongside TatB and C-
590 terminally His-tagged TatC were treated as described in A and equivalent volumes of the

591 elution fraction were analyzed by western blotting with anti-His, anti-TatB, or anti-FLAG
592 antibodies as indicated.

593

594 **Figure 7.** Multiple leucine substitutions in SufI signal peptide h-region partially re-route AmiA
595 to the Sec pathway. A - C. Overnight cultures of strain MC4100 Δ*amiA* Δ*amiC* Δ*tatABC*
596 harboring either pTH19kr (Δ*tat*) or pTAT101 producing wild type TatABC (*tat*$^+$) along with
597 pSUSufIss-mAmiA producing the indicated substitutions in the SufI signal peptide were sub-
598 cultured at 1:100 dilution and: A. grown for a further 3 hours at 37 °C, pelleted, re-suspended
599 to an OD_{600} of 0.1 and 8 μL of sample was spotted onto LB agar or LB agar containing 2%
600 SDS. Plates were incubated at 37 °C for 16 hours, or B. and C. supplemented with 0.5% SDS
601 (final concentration) and grown at 37 °C without shaking. The optical density at 600nm was
602 monitored every 20min using a plate reader. Error bars are ± SD, *n*=3 biological replicates.

603

604 **Figure 8.** The OmpA and DsbA signal peptides are able to functionally engage with the Tat
605 machinery. A. and B. Overnight cultures of strain MC4100 Δ*amiA* Δ*amiC* Δ*tatABC* harboring
606 either pTH19kr (Δ*tat*) or pTAT101 producing wild type TatABC (*tat*$^+$) along with a plasmid
607 encoding the indicated signal peptide fusion to AmiA were sub-cultured at 1:100 dilution,
608 supplemented with 0.5% SDS (final concentration) and grown at 37 °C without shaking. The
609 optical density at 600nm was monitored every 20min using a plate reader. Error bars are ±
610 SD, *n*=3 biological replicates. C. Cells of strain DADE co-producing TatB, C-terminally His-
611 tagged TatC and either C-terminally FLAG-tagged DsbA or OmpA, as indicated, were lysed
612 and incubated with digitonin and His-tagged TatC was isolated using Ni-charged beads.
613 Following elution of bound TatC-His, equivalent volumes of the eluate from each sample were
614 analyzed by western blotting with anti-His, anti-TatB, or anti-FLAG antibodies. D. An aliquot
615 cell lysate prior to digitonin treatment from part C. was ultracentrifuged to remove the cell
616 membranes. A small amount of the amount of the supernatant was retained as the input
617 fraction and the remainder was incubated with Ni-charged beads. The beads were washed

three times with wash buffer and aliquots of the input and eluate samples were analyzed by western blotting using an anti-FLAG antibody.

Figure S1. The SufIS12L substitution can act in *cis* to suppress inactivating substitutions in the SufI signal peptide twin-arginine motif. Overnight cultures of strain MC4100 Δ*amiA* Δ*amiC* Δ*tatABC* harboring A. pTH19kr alongside pSUSufIss-mAmiA (Δ*tat*) or pTAT101 (producing wild type TatABC) alongside unsubstituted pSUSufIss-mAmiA (*tat*$^{+}$), or B. – J. pTAT101 (producing wild type TatABC) alongside pSUSufIss-mAmiA encoding the indicated substitutions in the SufI signal peptide were sub-cultured at 1:100 dilution into LB supplemented with 0.5% SDS (final concentration) and grown at 37 °C without shaking The optical density at 600nm was monitored every 20min using a plate reader. Error bars are ± SD, *n*=3 biological replicates.

Figure S2. The twin-arginine substituted SufI^{S12L} signal peptide variants mediate strict Tat-dependent transport of AmiA. A and B. Overnight cultures of strain MC4100 Δ*amiA* Δ*amiC* Δ*tatABC* harboring either pTAT101 (producing wild type TatABC) alongside unsubstituted pSUSufIss-mAmiA (*tat*$^{+}$) or pTH19kr alongside either unsubstituted pSUSufIss-mAmiA (Δ*tat*) or pSUSufIss-mAmiA encoding the indicated substitutions in the SufI signal peptide as indicated were sub-cultured at 1:100 dilution and: A. grown for a further 3 hours at 37 °C, pelleted, re-suspended to an OD_{600} of 0.1 and 8 μL of sample was spotted onto LB agar or LB agar containing 2% SDS. Plates were incubated at 37 °C for 16 hours. or B. supplemented with 0.5% SDS (final concentration) and grown at 37 °C without shaking The optical density at 600nm was monitored every 20min using a plate reader. Error bars are ± SD, *n*=3 biological replicates.

Figure S3. Hydrophobic substitutions in the SufI signal peptide can rescue the transport defect of TatC^{E103K} and act in *cis* to suppress the SufI signal peptide R5K, R6K substitution. A.

Overnight cultures of strain MC4100 Δ*amiA* Δ*amiC* Δ*tatABC* harboring pTAT101 producing TatABC^{E103K} along with pSUSufIss-mAmiA producing the indicated substitution in the SufI signal peptide were sub-cultured at 1:100 dilution and grown for a further 3 hours at 37 °C, pelleted, re-suspended to an OD_{600} of 0.1 and 8 μL of sample was spotted onto LB agar or LB agar containing 2% SDS. Plates were incubated at 37 °C for 16 hours. B. Overnight cultures of MC4100 Δ*amiA* Δ*amiC* Δ*tatABC* harboring pTAT101 (producing wild type TatABC) alongside either unsubstituted pSUSufIss-mAmiA (WT) or pSUSufIss-mAmiA encoding the indicated substitutions in the SufI signal peptide were sub-cultured at 1:100 dilution in the presence of 0.5% SDS (final concentration) and grown at 37 °C without shaking The optical density at 600nm was monitored every 20min using a plate reader.

Figure S4. Analysis of SufI export mediated by signal peptide leucine substitutions in the TatC^{E103K} background and in a *tat* deletion strain. *E. coli* strain DADE co-producing his-tagged but otherwise native SufI or SufI with the indicated single leucine substitutions in the signal peptide (from a pQE80 plasmid) alongside either A. an empty plasmid vector (Δ*tat*) or B. wild type TatAB and TatC^{E103K} (from pTAT101) were grown to mid-log phase and fractionated into whole cell (upper panels) and periplasm (lower panels), then analyzed by Western blot with anti-6X His tag® or anti-RNA polymerase β subunit antibodies (cytoplasmic control protein. Strain DADE co-producing his-tagged but otherwise native SufI alongside either wild type TatABC (*tat*$^+$ WT SufI) or an empty vector (Δ*tat* WT SufI) were used as positive and negative controls, respectively. wc – whole cell. Equivalent volumes of sample were loaded for each of the whole cell samples, and for each of the periplasmic samples.

Figure S5. Detection of SufI-FLAG variants in input samples prior to Ni-affinity purification. Cells of strain DADE-P co-producing C-terminally FLAG-tagged SufI with its native signal peptide (WT SufI) or harboring the indicated leucine substitutions, alongside TatB and C-terminally His-tagged TatC or TatC^{F94D} were lysed and incubated with digitonin. 30 μL of the

672 digitonin solubilised fraction was mixed with an equal volume of 2x Laemmli buffer and 10 μL
673 of each sample was subject to SDS-PAGE (12% acrylamide) followed by western blot with
674 anti-FLAG antibody.

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Protein ID	Gene name	Uniprot Description	Signal sequence	Subcellular location	Likely Tat interaction?
>NP_414546.1	*yaaX*	Uncharacterized protein YaaX	MKKMQSIVLALSLVLVAPMAAQA	Periplasm	Y
>NP_414917.2	*phoA*	Alkaline phosphatase	MKQSTIALALLPLLFTPVTKA	Periplasm	
>NP_414554.1	*yaaI*	UPF0412 protein YaaI	MKSVFTISASLAISLMLCCTAQA	Periplasm	
>NP_414678.1	*yadK*	Uncharacterized fimbrial-like protein YadK	MHPTQRKLMKRIILFLSLLFCIACPAIA	Fimbrium	Y
>NP_414682.1	*yadV*	Probable fimbrial chaperone YadV	MFFNTKHTTALCFVTCMAFSSSSIA	Periplasm	
>NP_414683.1	*yadN*	Uncharacterized fimbrial-like protein YadN	MSKKLGFALSGLMLAMVAGTASA	Fimbrium	Y
>NP_414692.1	*fhuA*	Ferrichrome-iron receptor	MARSKTAQPKHSLRKIAVVVATAVSGMSVYAQA	OM	Y
>NP_414694.1	*fhuD*	Iron(3+)-hydroxamate-binding protein FhuD	MSGLPLISRRRLLTAMALSPLLWQMNTAHA	Periplasm	
>NP_414700.1	*btuF*	Vitamin B12-binding protein	MAKSLFRALVALSFLAPLWLNAA	Periplasm	
>NP_414703.1	*degP*	Periplasmic serine endoprotease DegP	MKKTTLALSALALSLGLALSPLSATA	Periplasm	Y
>NP_414719.1	*bamA*	Outer membrane protein assembly factor BamA	MAMKKLLIASLLFSSATVYG	OM	Y
>NP_414720.1	*skp*	Chaperone protein Skp	MKKWLLAAGLGLALATSAQA	Periplasm	Y
>NP_414734.1	*nlpE*	Lipoprotein NlpE	MVKKAIVTAMAVISLFTLMGC	OM	Y
>NP_414735.4	*yaeF*	Probable endopeptidase YaeF	MDKPKAYCRLFLPSFLLLSAC	Membrane	
>NP_414752.1	*yafT*	Uncharacterized lipoprotein YafT	MNSKKLCCICVLFSLLAGC	Membrane	Y
>NP_414755.1	*ivy*	Inhibitor of vertebrate lysozyme	MGRISSGGMMFKAITTVAALVIATSAMA	Periplasm	
>NP_414762.1	*yafL*	Probable endopeptidase YafL	MSLPSIPSFVLSGLLLICLPFSSFA	Periplasm	
>NP_414776.1	*phoE*	Outer membrane pore protein E	MKKSTLALVVMGIVASASVQA	OM	Y
>NP_414784.1	*ykfB*	Uncharacterized protein YkfB	MTILSLSRFMLAGVLLASFNASA	Periplasm	
>NP_414785.4	*yafY*	Lipoprotein YafY	MKRKTLPLLALVATTLFLIAC	IM	Y
>NP_414825.1	*ecpC*	Probable outer membrane usher protein EcpC	MPLRRFSPGLKAQFAFGMVFLFVQPDASA	Periplasm	N?
>NP_414827.1	*ecpA*	Common pilus major fimbrillin subunit EcpA	MKKKVLAIALVTVFTGMGVAQA	Fimbrium	Y
>NP_414858.1	*yahJ*	Uncharacterized protein YahJ	MKESNSRREFLSQSGKMVTA AALFGTSVPLAHA	Periplasm	Tat substrate
>NP_414863.1	*yahO*	Uncharacterized protein YahO	MKIISKMLVGALALAVTNVYA	Periplasm	
>NP_414899.2	*tauA*	Taurine-binding periplasmic protein	MAISSRNTLLAALAFIAFQAQA	Periplasm	Y
>NP_414910.1	*ampH*	D-alanyl-D-alanine-carboxypeptidase/endopeptidase AmpH	MKRSLLFSAVLCAASLTSVHA	Periplasm	Y
>NP_414918.4	*psiF*	Phosphate starvation-inducible protein PsiF	MKITLLVTLLFGLVFLTTVGA	Periplasm	
>NP_414946.4	*yajI*	Uncharacterized lipoprotein YajI	MNTNVFRLLLLGSLFSLSAC	Membrane	
>NP_414968.4	*yajG*	Uncharacterized lipoprotein YajG	MFKKILFPLVALFMLAGC	Membrane	Y
>NP_414976.1	*ybaV*	Uncharacterized protein YbaV	MKHGIKALLITLSLACAGMSHSALA	Periplasm	
>NP_414987.3	*ybaY*	Uncharacterized lipoprotein YbaY	MKLVHMASGLAVAIALA	OM	

>NP_414998.1	*mscK*	Mechanosensitive channel MscK	MTMFQYYKRSRHFVFSAFIAFVFVLLC	IM	Y
>NP_415013.1	*ushA*	Protein UshA	MKLLQRGVALALLTTFTLASETALA	Periplasm	
>NP_415027.1	*tesA*	Acyl-CoA thioesterase 1	MMNFNNVFRWHLPFLFLVLLTFRAAA	Periplasm	
>NP_415031.1	*ybbC*	Uncharacterized protein YbbC	MKYSSIFSMLSFFILFAC	Membrane	
>NP_415063.4	*sfmA*	Uncharacterized fimbrial-like protein SfmA	MKLRFISSALAAALFAATGSYA	Fimbrium	
>NP_415064.1	*sfmC*	Probable fimbrial chaperone SfmC	MMTKIKLLMLIIFYLIISASAHA	Periplasm	
>NP_415077.1	*ybcL*	UPF0098 protein YbcL	MKTLIVSTVLAFITFSAQAAA	Periplasm	
>NP_415100.1	*nfrA*	Bacteriophage adsorption protein A	MKENNLNRVIGWSGLLLTSLLSTSALA	OM	
>NP_415105.1	*cusF*	Cation efflux system protein CusF	MKKALQVAMFSLFTVIGFNAQA	Periplasm	Y
>NP_415116.1	*fepA*	Ferrienterobactin receptor	MNKKIHSLALLVNLGIYGVAQA	OM	Y
>NP_415124.1	*fepB*	Ferrienterobactin-binding periplasmic protein	MRLAPLYRNALLLTGLLLSGIA	Periplasm	Y
>NP_415137.2	*dsbG*	Thiol:disulfide interchange protein DsbG	MLKKILLLALLPAIAFA	Periplasm	Y
>NP_415144.1	*rna*	Ribonuclease I	MKAFWRNAALLAVSLLPFSSANA	Periplasm & Cytoplasm?	Y
>NP_415155.1	*pagP*	Lipid A palmitoyltransferase PagP	MNVSKYVAIFSFVFIQLISVGKVFANA	OM	
>NP_415166.1	*rlpA*	Endolytic peptidoglycan transglycosylase RlpA	MRKQWLGICIAAGMLAAC	Membrane	Y
>NP_415188.1	*gltI*	Glutamate/aspartate import solute-binding protein	MQLRKPATAILALALSAGLAQA	Periplasm	Y
>NP_415207.1	*chiP*	Chitoporin	MRTFSGKRSTLALAIAGVTAMSGFMA	OM	Y
>NP_415208.1	*chiQ*	Uncharacterized lipoprotein ChiQ	MKKLILIAIMASGLVAC	Membrane	Y
>NP_415215.1	*ybfP*	Uncharacterized lipoprotein YbfP	MKTNRSLVVIVSLITATLLLTAC	Membrane	
>NP_415228.1	*ybfA*	Uncharacterized protein YbfA	MELYREYPAWLIFLRRTYAVA	Periplasm	Y
>NP_415244.4	*ybgO*	Uncharacterized protein YbgO	MSAGKGLLLVICLLFLPLKSAMA	Fimbrium	
>NP_415245.1	*ybgP*	Uncharacterized fimbrial chaperone YbgP	MTFIKGLPLMLLTISLGCNA	Periplasm	
>NP_415246.2	*ybgQ*	Uncharacterized outer membrane usher protein YbgQ	MNIYRLSFVSCLVMAMPCAMA	OM	
>NP_415268.1	*tolB*	Protein TolB	MKQALRVAFGFLILWASVLHA	Periplasm	
>NP_415269.1	*pal*	Peptidoglycan-associated lipoprotein	MQLNKVLKGLMIALPVMAIAAC	OM	
>NP_415270.1	*cpoB*	Cell division coordinator CpoB	MSSNFRHQLLSLSLLVGIAAPWAAFA	Periplasm	Y
>NP_415274.1	*ybgS*	Uncharacterized protein YbgS	MKMTKLATLFLTATLSLASGAALA	Periplasm	
>NP_415284.1	*modA*	Molybdate-binding periplasmic protein	MARKWLNLFAGAALSFAVAGNA	Periplasm	Y
>NP_415293.1	*ybhC*	Putative acyl-CoA thioester hydrolase YbhC	MNTFSVSRLALALAFGVTLTAC	OM	
>NP_415323.1	*ybiJ*	Uncharacterized protein YbiJ	MKTINTVVAAMALSTLSFGVFA	Periplasm	
>NP_415326.1	*fiu*	Catecholate siderophore receptor Fiu	MENNRNFPARQFHSLTFFAGLCIGITPVAQAALA	OM	Y
>NP_415327.2	*mcbA*	Uncharacterized protein McbA	MKKCLTLLIATVLSGISLTAYA	Periplasm	Y

>NP_415332.1	*glnH*	Glutamine-binding periplasmic protein	MKSVLKVSLAALTLAFAVSSHA	Periplasm	
>NP_415335.1	*ompX*	Outer membrane protein X	MKKIACLSALAAVLAFTAGTSVA	OM	Y
>NP_415340.1	*ybiS*	Probable L,D-transpeptidase YbiS	MNMKLKTLFAAAFAVVGFCSTASA	Periplasm	
>NP_415351.1	*gsiB*	Glutathione-binding protein GsiB	MARAVHRSGLVALGIATALMASCAFA	Periplasm	
>NP_415355.1	*yliF*	Putative lipoprotein YliF	MSRINKFVLTVSLLIFIMISAVAC	Membrane	
>NP_415358.1	*ylil*	Aldose sugar dehydrogenase Ylil	MHRQSFFLVPLICLSSALWA	Periplasm	Y
>NP_415360.1	*dacC*	D-alanyl-D-alanine carboxypeptidase DacC	MTQYSSLLRGLAAGSAFLFLFAPTAFA	Periplasm	
>NP_415364.1	*ybjH*	Uncharacterized protein YbjH	MIMKNCLLLGALLMGFTGVAMA	Periplasm	
>NP_415375.1	*potF*	Putrescine-binding periplasmic protein	MTALNKKWLSGLVAGALMAVSVGTLA	Periplasm	Y
>NP_415381.1	*artJ*	ABC transporter arginine-binding protein 1	MKKLVLAALLASFTFGASA	Periplasm	Y
>NP_415384.1	*artI*	Putative ABC transporter arginine-binding protein 2	MKKVLIAALIAGFSLSATA	Periplasm	Y
>NP_415386.1	*ybjP*	Uncharacterized lipoprotein YbjP	MRYSKLTMLIPCALLLSAC	Membrane	
>NP_415411.2	*lolA*	Outer-membrane lipoprotein carrier protein	MKKIAITCALLSSLVASSVWA	Periplasm	Y
>NP_415446.1	*ycbK*	Uncharacterized protein YcbK	MDKFDANRRKLLALGGVALGAAILPTPAFA	Periplasm	Tat substrate
>NP_415449.1	*ompF*	Outer membrane protein F	MMKRNILAVIVPALLVAGTANA	OM	Y
>NP_415456.4	*ssuA*	Putative aliphatic sulfonates-binding protein	MRNIIKLALAGLLSVSTFAVA	Periplasm	Y
>NP_415458.2	*elfA*	Fimbrial subunit ElfA	MKKSVLTAFITVVCATSSVMA	Fimbrium	Y
>NP_415459.1	*elfD*	Probable fimbrial chaperone protein ElfD	MKTCITKGIVTVSLTAILLSCSSAWA	Periplasm	
>NP_415460.1	*elfC*	Probable outer membrane usher protein ElfC	MYRTHRQHSLLSSGGVPSFIGGLVVFVSAAFNAQA	OM	Y
>NP_415464.4	*ycbF*	Uncharacterized fimbrial chaperone YcbF	MTNTWNRLALLIFAVLSLLVAGELQA	Periplasm	
>NP_415472.2	*ymbA*	Uncharacterized lipoprotein YmbA	MKKWLVTIAALWLAGC	Membrane	Y
>NP_415477.1	*ompA*	Outer membrane protein A	MKKTAIAIAVALAGFATVAQA	OM	Y
>NP_415484.1	*yccT*	UPF0319 protein YccT	MKTGIVTTLIALCLPVSVFA	Periplasm	
>NP_415491.1	*hyaA*	Hydrogenase-1 small chain	MNNEETFYQAMRRQGVTRRSFLKYCSLAATSLGLGAGMAPKIAWA	IM	Tat substrate
>NP_415500.1	*appA*	Periplasmic AppA protein	MKAILIPFLSLLIPLTPQSAFA	Periplasm	
>NP_415503.1	*gfcE*	Putative polysaccharide export protein GfcE	MKKNIFKFSVLTLAVLSLTAC	OM	Y
>NP_415504.1	*gfcD*	Uncharacterized lipoprotein GfcD	MKKNSYLLSCLAIAVSSAC	Membrane	Y
>NP_415505.1	*gfcC*	Uncharacterized protein GfcC	MNKLQSYFIASVLYVMTPHAFA	Periplasm	
>NP_415506.1	*gfcB*	Uncharacterized lipoprotein GfcB	MRPLILSIFALFLAGC	Membrane	
>NP_415522.1	*agp*	Glucose-1-phosphatase	MNKTLIAAAVAGIVLLASNAQA	Periplasm	
>NP_415537.1	*efeO*	Iron uptake system component EfeO	MTINFRRNALQLSVAALFSSAFMANA	Periplasm	Tat substrate?
>NP_415538.1	*efeB*	Deferrochelatase/peroxidase EfeB	MQYKDENGVNEPSRRRLLKVIGALALAGSCPVAHA	Periplasm	Tat substrate
>NP_415542.1	*pgaB*	Poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase	MLRNGNKYLLMLVSIIMLTAC	OM	Y

>NP_415559.1	*csgB*	Minor curlin subunit	MKNKLLFMMLTILGAPGIAAA	Fimbrium	
>NP_415560.1	*csgA*	Major curlin subunit	MKLLKVAAIAAIVFSGSALA	Fimbrium	
>NP_415562.1	*ymdA*	Uncharacterized protein YmdA	MFRPFLNSLMLGSLFFPFIAIA	Periplasm	
>NP_415566.1	*mdoG*	Glucans biosynthesis protein G	MMKMRWLSAAVMLTLYTSSSWA	Periplasm	
>NP_415574.1	*yceI*	Protein YceI	MKKSLLGLTFASLMFSAGSAVA	Periplasm	Y
>NP_415590.1	*flgA*	Flagella basal body P-ring formation protein FlgA	MLIIKRSVAIIAILFSPLSTA	Periplasm	Y
>NP_415598.3	*flgI*	Flagellar P-ring protein	MIKFLSALILLLVTTAAQA	Periplasm	
>NP_415620.1	*fhuE*	FhuE receptor	MLSTQFNRDNQYQAITKPSLLAGCIALALLPSAAFA	OM	N?
>NP_415630.1	*bhsA*	Multiple stress resistance protein BhsA	MKNVKTLIAAAILSSMSFASFA	OM	
>NP_415631.1	*ycfS*	Probable L,D-transpeptidase YcfS	MMIKTRFSRW LTFFTFAAAVALA	Periplasm	
>NP_415641.1	*potD*	Spermidine/putrescine-binding periplasmic protein	MKKWSRHLLAAGALALGMSAAHA	Periplasm	Y
>NP_415696.1	*pliG*	Inhibitor of g-type lysozyme	MKIKSIRKAVLLLALLTSTSFA	Periplasm	Y
>NP_415715.1	*treA*	Periplasmic trehalase	MKSPAPSRPQKMALIPACIFLCFAALSVQA	Periplasm	
>NP_415738.2	*ychO*	Uncharacterized protein YchO	MSRFVPRIIPFYLLLLVAGGTANA	Periplasm	
>NP_415759.1	*oppA*	Periplasmic oligopeptide-binding protein	MTNITKRSLVAAGVLAALMAGNVALA	Periplasm	Y
>NP_415772.1	*ompW*	Outer membrane protein W	MKKLTVAALAVTTLLSGSAFA	OM	Y
>NP_415799.1	*osmB*	Osmotically-inducible lipoprotein B	MFVTSKKMTAAVLAITLAMSLSAC	Membrane	Y
>NP_415810.1	*sapA*	Probable ABC transporter periplasmic-binding protein SapA	MRQVLSSLLVIAGLVSGQAIA	Periplasm	Y
>NP_415826.1	*ycjN*	Putative ABC transporter periplasmic-binding protein YcjN	MIKSKIVLLSALVSCALIS	Periplasm	
>NP_415835.1	*ompG*	Outer membrane protein G	MKKLLPCTALVMCAGMACAQA	OM	Y
>NP_415895.1	*ompN*	Outer membrane protein N	MKSKVLALLIPALLAAGAAHA	OM	
>NP_415936.1	*ydcA*	Uncharacterized protein YdcA	MKKLALILFMGTLVSFYADA	Periplasm	Y
>NP_415941.5	*mdoD*	Glucans biosynthesis protein D	MDRRRFIKGSMAMAAVCGTSGIASLFSQAAFA	Periplasm	Tat substrate
>NP_415948.1	*ydcL*	Uncharacterized lipoprotein YdcL	MRTTSFAKVAALCGLLALSGC	Membrane	
>NP_415953.1	*yncJ*	Uncharacterized protein YncJ	MFTKALSVVLLTCALFSGQLMA	Periplasm	
>NP_415957.1	*ydcS*	Putative ABC transporter periplasmic-binding protein YdcS	MSKTFARSSLCALSMTIMTAHA	Periplasm	
>NP_415969.1	*yncE*	Uncharacterized protein YncE	MHLRHLFSSRLRGSLLLGSLLVVSSFSTQA	Periplasm	N?
>NP_415991.1	*fdnG*	Formate dehydrogenase, nitrate-inducible, major subunit	MDVSRRQFFKICAGGMAGTTVAALGFAPKQALA	Periplasm	Tat substrate
>NP_416004.1	*ddpA*	Probable D,D-dipeptide-binding periplasmic protein DdpA	MKRSISFRPTLLALVLATNFPVAHA	Periplasm	N?
>NP_416008.1	*yddW*	UPF0748 lipoprotein YddW	MDICSRNKKLTIRRPAILVALALLLCSC	Membrane	Y
>NP_416015.2	*ydeN*	Uncharacterized sulfatase YdeN	MKSALKKSVVSTSISLILASGMAAFAAHA	Periplasm	N?
>NP_416054.1	*ydeI*	Uncharacterized protein YdeI	MKFQAIVLASFLVMPYALA	Periplasm	

>NP_416100.1	*ynfB*	UPF0482 protein YnfB	MKITLSKRIGLLAILLPCALALSTTVHA	Periplasm	Y
>NP_416103.2	*ynfD*	Uncharacterized protein YnfD	MKLSTCCAALLLALASPAVLA	Periplasm	
>NP_416114.2	*asr*	Acid shock protein	MKKVLALVVAAAMGLSSAAFA	Periplasm	Y
>NP_416121.1	*ydgH*	Protein YdgH	MKLKNTLLASALLSAMAFSVNA	Periplasm	
>NP_416132.2	*uidC*	Membrane-associated protein UidC	MRKIVAMAVICLTAASGLTSAYA	OM?	Y
>NP_416163.1	*sodC*	Superoxide dismutase [Cu-Zn]	MKRFSLAILALVVATGAQA	Periplasm	Y
>NP_416172.1	*mepH*	Murein DD-endopeptidase MepH	MARINRISITLCALLFTTLPLTPMAHA	Periplasm	
>NP_416192.1	*lpp*	Major outer membrane lipoprotein Lpp	MKATKLVLGAVILGSTLLAGC	OM	
>NP_416193.1	*ynhG*	Probable L,D-transpeptidase YnhG	MKRASLLTLTLIGAFSAIQAAWA	Periplasm	Y
>NP_416223.1	*nlpC*	Probable endopeptidase NlpC	MRFCLILITALLLAGC	Membrane	
>NP_416236.1	*ydiY*	Uncharacterized protein YdiY	MKLLKTVPAIVMLAGGMFASLNAAA	OM	
>NP_416257.1	*spy*	Periplasmic chaperone Spy	MRKLTALFVASTLALGAANLAHA	Periplasm	Y
>NP_416274.1	*ynjH*	Uncharacterized protein YnjH	MSRALFAVVLAFPLIALA	Periplasm	
>NP_416296.1	*mipA*	MltA-interacting protein	MTKLKLLALGVLIATSAGVAHA	OM	
>NP_416355.1	*yobA*	Protein YobA	MASTARSLRYALAILTTSLVTPSVWAHA	Periplasm	
>NP_416371.4	*znuA*	High-affinity zinc uptake system protein ZnuA	MLHKKTLLFAALSAALWGGA	Periplasm	Y
>NP_416414.1	*araF*	L-arabinose-binding periplasmic protein	MHKFTKALAAIGLAAVMSQSAMA	Periplasm	
>NP_416438.1	*yedD*	Uncharacterized lipoprotein YedD	MKKLAIAGALLLLAGC	Membrane	Y
>NP_416482.1	*zinT*	Metal-binding protein ZinT	MAIRLYKLAVALGVFIVSAPAFS	Periplasm	
>NP_416547.1	*wcaM*	Colanic acid biosynthesis protein WcaM	MPFKKLSRRTFLTASSALAFLHTPFARA	Periplasm	Tat substrate
>NP_416578.2	*mdtA*	Multidrug resistance protein MdtA	MKGSYKSRWVIVIVVVIAAIA	Periplasm	
>NP_416610.4	*rcnB*	Nickel/cobalt homeostasis protein RcnB	MTIKNKMLLGALLLVTSAAWAAPA	Periplasm	
>NP_416612.1	*yehB*	Outer membrane usher protein YehB	MLRMTPLASAIVALLLGIEAYA	OM	
>NP_416613.1	*yehC*	Probable fimbrial chaperone YehC	MAAIPWRPFNLRGIKMKGLLSLLIFSMVLPAHA	Periplasm	
>NP_416627.2	*yehR*	Uncharacterized lipoprotein YehR	MKAFNKLFSLVVASVLVFSLAGC	Membrane	
>NP_416636.1	*bglX*	Periplasmic beta-glucosidase	MKWLCSVGIAVSLALQPALA	Periplasm	
>NP_416638.4	*pbpG*	D-alanyl-D-alanine endopeptidase	MPKFRVSLFSLALMLAVPFAPQAVA	Periplasm	
>NP_416655.1	*mglB*	D-galactose-binding periplasmic protein	MNKKVLTLSAVMASMLFGAA	Periplasm	Y
>NP_416682.4	*yejA*	Uncharacterized protein YejA	MIVRILLLFIALFTFGVQAQA	Periplasm	
>NP_416698.1	*ccmH*	Cytochrome c-type biogenesis protein CcmH	MRFLLGVLMLMISGSALA	Periplasm	
>NP_416710.1	*napA*	Periplasmic nitrate reductase	MKLSRRSFMKANAVAAAAAAAGLSVPGVARA	Periplasm	Tat substrate
>NP_416713.1	*eco*	Ecotin	MKTILPAVLFAAFATTSAWA	Periplasm	
>NP_416719.1	*ompC*	Outer membrane protein C	MKVKVLSLLVPALLVAGAANA	OM	
>NP_416742.1	*glpQ*	Glycerophosphodiester phosphodiesterase, periplasmic	MKLTLKNLSMAIMMSTIVMGSSAMA	Periplasm	
>NP_416812.1	*hisJ*	Histidine-binding periplasmic protein	MKKLVLSLSLVLAFSSATA	Periplasm	Y

>NP_416835.2	*yfcO*	Uncharacterized protein YfcO	MKILRWLFALVMLIATTEAMA	OM	
>NP_416837.1	*yfcQ*	Uncharacterized fimbrial-like protein YfcQ	MRKTFLTLLCVSSAIAHA	Fimbrium	Y
>NP_416838.1	*yfcR*	Uncharacterized fimbrial-like protein YfcR	MTGGVMSQKFVVGAGLLVCSVCSLSAMA	Fimbrium	
>NP_416839.1	*yfcS*	Probable fimbrial chaperone YfcS	MSDLLCSAKLGAMTLALLLSATSLSALA	Periplasm	
>NP_416891.1	*ypeC*	Uncharacterized protein YpeC	MFRSLFLAAALMAFTPLAANA	Periplasm	
>NP_416930.1	*amiA*	N-acetylmuramoyl-L-alanine amidase AmiA	MSTFKPLKTLTSRRQVLKAGLAALTLSGMSQAIA	Periplasm	Tat substrate
>NP_416944.1	*yffR*	Uncharacterized protein YffR	MKVLGNILWWAFVGFMAYA	Periplasm	
>NP_416989.1	*bepA*	Beta-barrel assembly-enhancing protease	MFRQLKKNLVATLIAAMTIGQVAPAFA	Periplasm	Y
>NP_417000.1	*yfgH*	Uncharacterized lipoprotein YfgH	MMKFKKCLLPVAMLASFTLAGC	OM	Y
>NP_417007.1	*bamB*	Outer membrane protein assembly factor BamB	MQLRKLLLPGLLSVTLLSGC	OM	Y
>NP_417043.1	*yphF*	ABC transporter periplasmic-binding protein YphF	MPTKMRTTRNLLLMATLLGSALFARA	Periplasm	Y
>NP_417066.1	*rseB*	Sigma-E factor regulatory protein RseB	MKQLWFAMSLVTGSLLFSANASA	Periplasm	
>NP_417086.1	*bamD*	Outer membrane protein assembly factor BamD	MTRMKYLVAAATLSLFLAGC	OM	
>NP_417125.1	*yfjT*	Uncharacterized protein YfjT	MKIRSLSRFVLASTMFASFTASA	Periplasm	
>NP_417289.2	*ygdI*	Uncharacterized lipoprotein YgdI	MKKTAAIISACMLTFALSAC	Membrane	Y
>NP_417294.4	*amiC*	N-acetylmuramoyl-L-alanine amidase AmiC	MSGSNTAISRRRLLQGAGAMWLLSVSQVSLA	Periplasm	Tat substrate
>NP_417369.1	*dsbC*	Thiol:disulfide interchange protein DsbC	MKKGFMLFTLLAAFSGFAQA	Periplasm	Y
>NP_417420.1	*endA*	Endonuclease-1	MYRYLSIAAVVLSAAFSGPALA	Periplasm	
>NP_417432.1	*ansB*	L-asparaginase 2	MEFFKKTALAALVMGFSGAALA	Periplasm	Y
>NP_417445.1	*yghG*	Uncharacterized lipoprotein YghG	MSIKQMPGRVLISLLLSVTGLLSGC	Membrane	
>NP_417470.1	*hybA*	Hydrogenase-2 operon protein HybA	MNRRNFIKAASCGALLTGALPSVSHAAA	Periplasm	Tat substrate
>NP_417471.1	*hybO*	Hydrogenase-2 small chain	MTGDNTLIHSHGINRRDFMKLCAALAATMGLSSKAAA	Periplasm	Tat substrate
>NP_417489.1	*ftsP/sufI*	Cell division protein FtsP/SufI	MSLSRRQFIQASGIALCAGAVPLKASA	Periplasm	Tat substrate
>NP_417492.1	*ygiS*	Probable deoxycholate-binding periplasmic protein YgiS	MYTRNLLWLVSLVSAAPLYA	Periplasm	Y
>NP_417496.1	*ygiW*	Protein YgiW	MKKFAAVIAVMALCSAPVMA	Periplasm	Y
>NP_417520.1	*yqiI*	Uncharacterized protein YqiI	MRYLLIVITFFMGFSSLPAWA	Fimbrium	
>NP_417551.1	*ygjK*	Glucosidase YgjK	MKIKTILTPVTCALLISFSAHA	Periplasm	
>NP_417611.1	*yraH*	Uncharacterized fimbrial-like protein YraH	MNKVTKTAIAGLLALFAGNAAA	Fimbrium	
>NP_417612.1	*yraI*	Probable fimbrial chaperone YraI	MSKRTFAVILTLLCSFCIGQALA	Periplasm	Y
>NP_417614.1	*yraK*	Uncharacterized fimbrial-like protein YraK	MKRAPLITGLLLISTSCAYA	Fimbrium	Y
>NP_417659.1	*mlaC*	Probable phospholipid-binding protein MlaC	MFKRLMMVALLVIAPLSAATA	Periplasm	Y
>NP_417667.1	*lptA*	Lipopolysaccharide export system protein LptA	MKFKTNKLSLNLVLASSLLAASIPAFA	Periplasm	

>NP_417681.1	*gltF*	Protein GltF	MFFKKNLTTAAICAALSVAAFSAMA	Periplasm	Y
>NP_417683.1	*yhcD*	Uncharacterized outer membrane usher protein YhcD	MLKKTLLAYTIGFAFSPPANA	OM	Y
>NP_417686.1	*yhcF*	Uncharacterized protein YhcF	MNNVKLLIAGSAFFAMSAQA	Cytoplasm/ Periplasm?	
>NP_417701.1	*degQ*	Periplasmic pH-dependent serine endoprotease DegQ	MKKQTQLLSALALSVGLTLSASFQAVA	Periplasm	Y
>NP_417705.2	*yhcN*	Uncharacterized protein YhcN	MKIKTTVAALSVLSVLSFGAFA	Periplasm	
>NP_417797.1	*chiA*	Probable bifunctional chitinase/lysozyme	MKLNIFTKSMIGMGLVCSALPALA	Periplasm	
>NP_417806.1	*fkpA*	FKBP-type peptidyl-prolyl cis-trans isomerase FkpA	MKSLFKVTLLATTMAVALHAPITFAAEA	Periplasm	
>NP_417822.1	*ppiA*	Peptidyl-prolyl cis-trans isomerase A	MFKSTLAAMAAVFALSALSPAAMA	Periplasm	
>NP_417904.1	*ggt*	Gamma-glutamyltranspeptidase	MIKPTFLRRVAIAALLSGSCFSAAA	Periplasm	Y
>NP_417910.1	*ugpB*	sn-glycerol-3-phosphate-binding periplasmic protein UgpB	MKPLHYTASALALGLALMGNAQA	Periplasm	
>NP_417915.1	*livK*	Leucine-specific-binding protein	MKRNAKTIIAGMIALAISHTAMA	Periplasm	Y
>NP_417933.1	*nikA*	Nickel-binding periplasmic protein	MLSTLRRTLFALLACASFIVHAAA	Periplasm	Y
>NP_417963.4	*slp*	Outer membrane protein slp	MNMTKGALILSLSFLLAAC	OM	
>NP_417966.4	*hdeB*	Acid stress chaperone HdeB	MNISSLRKAFIFMGAVAALSLVNAQSALA	Periplasm	Y
>NP_417984.1	*yhjJ*	Protein YhjJ	MQGTKIRLLAGGLLMMATAGYVQA	Periplasm	
>NP_418023.1	*xylF*	D-xylose-binding periplasmic protein	MKIKNILLTLCTSLLLTNVAAHA	Periplasm	
>NP_418028.1	*malS*	Periplasmic alpha-amylase	MKLAACFLTLLPGFAVA	Periplasm	
>NP_418041.1	*yiaT*	Putative outer membrane protein YiaT	MLINRNIVALFALPFMASATA	OM	Y
>NP_418070.6	*envC*	Murein hydrolase activator EnvC	MTRAVKPRRFAIRPIIYASVLSAGVLLCAFSAHA	Periplasm	Y
>NP_418117.1	*nlpA*	Lipoprotein 28	MKLTTHHLRTGAALLLAGILLAGC	IM	
>NP_418184.1	*pstS*	Phosphate-binding protein PstS	MKVMRTTVATVVAATLSMSAFSVFAEA	Periplasm	
>NP_418207.1	*rbsB*	Ribose import binding protein RbsB	MNMKKLATLVSAVALSATVSANAMA	Periplasm	Y
>NP_418245.1	*aslA*	Arylsulfatase	MEFSFSPKRLVVAVAAALPLMASA	Periplasm	Y
>NP_418297.1	*dsbA*	Thiol:disulfide interchange protein DsbA	MKKIWLALAGLVLAFSASA	Periplasm	Y
>NP_418311.1	*ompL*	Porin OmpL	MKKINAIILLSSLTSASVFAGA	OM	Y
>NP_418355.1	*yiiQ*	Uncharacterized protein YiiQ	MKPGCTLFFLLCSALTVTTEAHA	Periplasm	
>NP_418372.1	*yiiX*	Uncharacterized protein YiiX	MKNRLLILSLLVSVPAFA	Periplasm	
>NP_418401.1	*btuB*	Vitamin B12 transporter BtuB	MIKKASLLTACSVTAFSAWA	OM	Y
>NP_418430.2	*zraP*	Zinc resistance-associated protein	MKRNTKIALVMMALSAMAMGSTSAFA	Periplasm	Y
>NP_418450.1	*yjbE*	Uncharacterized protein YjbE	MKKVLYGIFAISALAATSAWA	Periplasm	Y
>NP_418452.1	*yjbG*	Uncharacterized protein YjbG	MIKQTIVALLLSVGASSVFA	Periplasm	
>NP_418458.1	*malE*	Maltose-binding periplasmic protein	MKIKTGARILALSALTTMMFSASA	Periplasm	
>NP_418460.1	*lamB*	Maltoporin	MMITLRKLPLAVAVAAGVMSAQAMA	OM	Y

>NP_418461.1	*malM*	Maltose operon periplasmic protein	MKMNKSLIVLCLSAGLLASA	Periplasm	
>NP_418479.1	*aphA*	Class B acid phosphatase	MRKITQAISAVCLLFALNSSAVA	Periplasm	Y
>NP_418495.2	*nrfB*	Cytochrome c-type protein NrfB	MSVLRSLLTAGVLASGLLWSLNGITATPAAQA	Periplasm	
>NP_418499.1	*nrfF*	Formate-dependent nitrite reductase complex subunit NrfF	MNKGLLTLLLLFTCFAHA	Periplasm	
>NP_418502.1	*yjcO*	Uncharacterized protein YjcO	MKKIIALMLFLTFFAHA	Periplasm	Y
>NP_418507.2	*yjcS*	Putative alkyl/aryl-sulfatase YjcS	MNNSRLFRLSRIVIALTAASGMMVNTANA	Periplasm	
>NP_418512.1	*alsB*	D-allose-binding periplasmic protein	MNKYLKYFSGTLVGLMLSTSAFA	Periplasm	
>NP_418529.1	*phnD*	Phosphonates-binding periplasmic protein	MNAKIIASLAFTSMFSLSTLLSPAHA	Periplasm	
>NP_418620.1	*yjfY*	Uncharacterized protein YjfY	MFSRVLALLAVLLLSANTWA	Periplasm	
>NP_418641.1	*tamA*	Translocation and assembly module TamA	MRYIRQLCCVSLLCLSGSAVA	OM	Y
>NP_418648.1	*ytfQ*	ABC transporter periplasmic-binding protein YtfQ	MWKRLLIVSAVSAAMSSMALA	Periplasm	Y
>NP_418710.4	*fecB*	Fe(3+) dicitrate-binding periplasmic protein	MLAFIRFLFAGLLLVISHAFA	Periplasm	
>NP_418711.1	*fecA*	Fe(3+) dicitrate transport protein FecA	MTPLRVFRKTTPLVNTIRLSLLPLAGLSFSAFA	OM	N?
>NP_418730.4	*nanM*	N-acetylneuraminate epimerase	MNKTITALAIMMASFAANA	Periplasm	
>NP_418734.1	*fimA*	Type-1 fimbrial protein, A chain	MKIKTLAIVVLSALSLSSTAALA	Fimbrium	
>NP_418735.2	*fimI*	Fimbrin-like protein FimI	MKRKRLFLLASLLPMFALA	Fimbrium	Y
>NP_418736.3	*fimC*	Chaperone protein FimC	MSNKNVNVRKSQEITFCLLAGILMFMAMMVAGRAEA	Periplasm	N?
>NP_418737.1	*fimD*	Outer membrane usher protein FimD	MSYLNLRLYQRNTQCLHIRKHRLAGFFVRLVVACAFAAQAPLSSA	OM	Y
>NP_418738.1	*fimF*	Protein FimF	MRNKPFYLLCAFLWLAVSHALA	Fimbrium	Y
>NP_418739.1	*fimG*	Protein FimG	MKWCKRGYVLAAILALASATIQA	Fimbrium	Y
>NP_418793.1	*osmY*	Osmotically-inducible protein Y	MTMTRLKISKTLLAVMLTSAVATGSAYA	Periplasm	
>NP_418809.4	*slt*	Soluble lytic murein transglycosylase	MEKAKQVTWRLLAAGVCLLTVSSVARA	Periplasm	
>NP_418814.1	*creA*	Protein CreA	MKYKHLILSLSLIMLGPLAHA	Periplasm	
>YP_001165332.1	*yjbT*	Uncharacterized protein YjbT	MKRNLIKVVKMKPYFAALMLSVSVLPAYA	Periplasm	N?
>YP_002791241.1	*rzoQ*	Putative lipoprotein RzoQ	MRNRNLLKFLPGLLICLIVLTSC	Membrane	Y
>YP_026195.1	*yqhG*	Uncharacterized protein YqhG	MKIILLFLAALASFTVHA	Periplasm	
>YP_026223.1	*livJ*	Leu/Ile/Val-binding protein	MNIKGKALLAGCIALAFSNMALA	Periplasm	
>YP_026240.1	*yidX*	Uncharacterized protein YidX	MKLNFKGFFKAAGLFPLALMLSGC	Membrane	
>YP_026277.1	*cpxP*	Periplasmic protein CpxP	MRIVTAAVMASTLAVSSLSHA	Periplasm	
>YP_026281.1	*yjdP*	Uncharacterized protein YjdP	MKRFPLFLLFTLLTLSTVPAQA	Periplasm	Y
>YP_026283.1	*ecnA*	Entericidin A	MMKRLIVLVLLASTLLTGC	Membrane	Y
>YP_588473.1	*yifL*	Uncharacterized lipoprotein YifL	MKNVFKALTVLLTLFSLTGC	Membrane	

Table S1. *E. coli* proteins with known or predicted signal peptides. Protein sequences encoded by *E. coli* MG1655 were retrieved from Uniprot (www.uniprot.org) and analyzed for the presence of a signal peptide using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP). The n-regions were analyzed manually; twin arginines are colored red, twin lysines green, lys-arg or arg-lys are shown in blue. Pairings of Arg-Asn, Arg-Asp, Arg-Gln, Arg-Glu and Arg-His (which from this study can be suppressed by increased signal peptide hydrophobicity) are shown in orange. For those signal peptides harboring any of these dipeptides in their n-regions the likely location of the h-region is shown in underline. Pairings within five residues of the h-region were considered likely to interact with the Tat pathway. Known or probable Tat substrates are shown in yellow highlight.

Plasmid	Description	Reference
pTAT101	Low copy number vector producing TatABC under the control of *tat* promoter. Kan^r	(37)
pTH19kr	Low copy-number cloning vector. Backbone of pTAT101	(62)
pTAT101CP48L	As pTAT101, TatC P48L exchange	(18)
pTAT101CF94D	As pTAT101, TatC F94D exchange	(27)
pTAT101C103K	As pTAT101, TatC E103K exchange	(37)
pTAT101CV145E	As pTAT101, TatC V145E exchange	(18)
pTAT101CQ215R	As pTAT101, TatC Q215R exchange	(37)
pSU18	Medium copy vector. Cm^r	(63)
pSUSufIss-mAmiA	pSU18, carrying SufIss-mAmiA	(27)
pSUSufIssA11L-mAmiA	As pSUSufIss-mAmiA, SufIss A11L exchange	This work
pSUSufIssS12L-mAmiA	As pSUSufIss-mAmiA, SufIss S12L exchange	This work
pSUSufIssG13L-mAmiA	As pSUSufIss-mAmiA, SufIss G13L exchange	This work
pSUSufIssA15L-mAmiA	As pSUSufIss-mAmiA, SufIss A15L exchange	This work
pSUSufIssA18L-mAmiA	As pSUSufIss-mAmiA, SufIss A18L exchange	This work
pSUSufIssG19L-mAmiA	As pSUSufIss-mAmiA, SufIss G19L exchange	This work
pSUSufIssA20L-mAmiA	As pSUSufIss-mAmiA, SufIss A20L exchange	This work
pSUSufIssRD-mAmiA	As pSUSufIss-mAmiA, SufIss R6D exchange	This work
pSUSufIssREmAmiA	As pSUSufIss-mAmiA, SufIss R6E exchange	This work
pSUSufIssRH-mAmiA	As pSUSufIss-mAmiA, SufIss R6H exchange	This work
pSUSufIssRNmAmiA	As pSUSufIss-mAmiA, SufIss R6N exchange	This work
pSUSufIssRQ-mAmiA	As pSUSufIss-mAmiA, SufIss R6Q exchange	This work
pSUSufIssKHmAmiA	As pSUSufIss-mAmiA, SufIss R5K, R6H exchange	This work
pSUSufIssKQ-mAmiA	As pSUSufIss-mAmiA, SufIss R5K, R6Q exchange	This work
pSUSufIssKKmAmiA	As pSUSufIss-mAmiA, SufIss R5K, R6K exchange	This work
pSUSufIssHHmAmiA	As pSUSufIss-mAmiA, SufIss R5H, R6H exchange	This work
pSUSufIssRD-S12L - mAmiA	As pSUSufIssRD-mAmiA, SufIss S12L exchange	This work
pSUSufIssRE-S12L mAmiA	As pSUSufIssRE-mAmiA, SufIss S12L exchange	This work
pSUSufIssRH-S12L - mAmiA	As pSUSufIssRH-mAmiA, SufIss S12L exchange	This work
pSUSufIssRN-S12L mAmiA	As pSUSufIssRN-mAmiA, SufIss S12L exchange	This work
pSUSufIssRQ-S12L - mAmiA	As pSUSufIssRQ-mAmiA, SufIss S12L exchange	This work
pSUSufIssKH-S12L mAmiA	As pSUSufIssKH-mAmiA, SufIss S12L exchange	This work
pSUSufIssKQ-S12L - mAmiA	As pSUSufIssKQ-mAmiA, SufIss S12L exchange	This work
pSUSufIssKK-S12L mAmiA	As pSUSufIssKK-mAmiA, SufIss S12L exchange	This work
pSUSufIssHH-S12L mAmiA	As pSUSufIssHH-mAmiA, SufIss S12L exchange	This work
pSUSufIssKK-G13L mAmiA	As pSUSufIssKK-mAmiA, SufIss G13L exchange	This work

pSUSuflssKK-A15L mAmiA	As pSUSuflssKK-mAmiA, Suflss A15L exchange	This work
pSUSuflssKK-G19L mAmiA	As pSUSuflssKK-mAmiA, Suflss G19L exchange	This work
pSUSuflssS12LG13L-mAmiA	As pSUSuflss-mAmiA, Suflss S12L, G13L exchange	This work
pSUSuflssS12LG13L4L15L-mAmiA	As pSUSuflss-mAmiA, Suflss S12L, G13L, I14L, A15L exchange	This work
pSUSuflssS17LS18L19L20L-mAmiA	As pSUSuflss-mAmiA, Suflss C17L, A18L, G19L, A20L exchange	This work
pSUDsbAss-mAmiA	As pSUSuflss-mAmiA, *suflss* substituted with *dsbAss*	This work
pSUDsbAssi16K-mAmiA	As pSUDsbAss-mAmiA, DsbAss 16K insertion	This work
pSUOmpAss-mAmiA	As pSUSuflss-mAmiA, *suflss* substituted with *ompAss*	This work
pSUOmpAssi18K-mAmiA	As pSUOmpAss-mAmiA, OmpAss 18K insertion	This work
pQE80-Suflhis	pQE80 carrying $sufI_{his}$	(27)
pQE80-Suflhis-S12L	As pQE80-Suflhis, SufI S12L exchange	This work
pQE80-Suflhis-G13L	As pQE80-Suflhis, SufI S13L exchange	This work
pQE80-Suflhis-A15L	As pQE80-Suflhis, SufI S15L exchange	This work
pQE80-Suflhis-G19L	As pQE80-Suflhis, SufI S19L exchange	This work
pQE80-Suflhis-S12LG13L	As pQE80-Suflhis, SufI S12L, G13L exchange	This work
pQE80-Suflhis-12L13L14L15L	As pQE80-Suflhis, SufI S12L, G13L, I14L, A15L exchange	This work
pQE80-Suflhis-17L18L19L20L	As pQE80-Suflhis, SufI C17L, A18L, G19L, A20L exchange	This work
pFAT75ΔA -Suflhis	*tatBC* with $sufI_{his}$ in pQE60	(18)
pFAT75ΔA-SuflFLAG	As pFAT75ΔA-Suflhis, *suflhis* substituted with *sufl*FLAG	This work
pFATBChis-SuflFLAG	As pFAT75ΔA-SuflFLAG, *tatC* his-tagged	This work
pFATBChis-SuflS12LFLAG	As pFATBChis-SuflFLAG, SufI S12L exchange	This work
pFATBChis-SuflG13LFLAG	As pFATBChis-SuflFLAG, SufI G13L exchange	This work
pFATBChis-SuflA15LFLAG	As pFATBChis-SuflFLAG, SufI A15L exchange	This work
pFATBChis-SuflG19LFLAG	As pFATBChis-SuflFLAG, SufI G19L exchange	This work
pFATBC94Dhis-SuflFLAG	As pFATBChis-SuflFLAG, TatC F94D exchange	This work
pFATBC94Dhis-SuflS12LFLAG	As pFATBChis-SuflS12LFLAG, TatC F94D exchange	This work
pFATBC94Dhis-SuflG13LFLAG	As pFATBChis-SuflG13LFLAG, TatC F94D exchange	This work
pFATBC94Dhis-SuflA15LFLAG	As pFATBChis-SuflA15LFLAG, TatC F94D exchange	This work
pFATBC94Dhis-SuflG19LFLAG	As pFATBChis-SuflG19LFLAG, TatC F94D exchange	This work
pFATBC94Dhis-SufI12L13L14L15LFLAG	As pFATBChis-SuflFLAG, SufI S12L, G13L, I14L, A15L exchange	This work

pFATBC94Dhis-SufI17L18L19L20LFLAG	As pFATBChis-SufIFLAG, SufI C17L, A18L, G19L, A20L exchange	This work
pFATBChis-OmpAssi18KSufIFLAG	As pFATBChis-OmpAssSufIFLAG, OmpA 18K insertion	This work
pQEBChis-OmpAFLAG	pQE80 coproducing TatBChis and OmpAFLAG	This work
pQEBChis-DsbAFLAG	pQE80 coproducing TatBChis and DsbAFLAG	This work

Table S2. Plasmids used and constructed in this study.

SuflA11L-F	GTCAGTTCATTCAGCTGTCGGGGATTGCAC
SuflA11L-R	GTGCAATCCCCGACAGCTGAATGAACTGAC
Sufl S12L-F	GTTCATTCAGGCACTGGGGATTGCACTTTG
Sufl S12L-R	CAAAGTGCAATCCCCAGTGCCTGAATGAAC
SuflG13L-F	GTTCATTCAGGCATCGCTGATTGCACTTTGTGC
SuflG13L-R	GCACAAAGTGCAATCAGCGATGCCTGAATGAAC
SuflA15L-F	CAGGCATCGGGGATTCTGCTTTGTGCAGGCGC
SuflA15L-R	GCGCCTGCACAAAGCAGAATCCCCGATGCCTG
SuflA18L-F	GGGATTGCACTTTGTCTGGGCGCTGTTCCCCTG
SuflA18L-R	CAGGGGAACAGCGCCCAGACAAAGTGCAATCCC
SuflG19L-F	GATTGCACTTTGTGCACTGGCTGTTCCCCTGAAG
SuflG19L-R	CTTCAGGGGAACAGCCAGTGCACAAAGTGCAATC
SuflA20L-F	GCACTTTGTGCAGGCCTGGTTCCCCTGAAGGCC
SuflA20L-R	GGCCTTCAGGGGAACCAGGCCTGCACAAAGTGC
Sufl S12LG13L-F	GTTCATTCAGGCACTGCTGATTGCACTTTGTGC
Sufl S12LG13L-R	GCACAAAGTGCAATCAGCAGTGCCTGAATGAAC
Sufl S12LG13L14L15L-F	CATTCAGGCACTGCTGCTGCTGCTTTGTGCAGGCGCTG
Sufl S12LG13L14L15L-R	CAGCGCCTGCACAAAGCAGCAGCAGCAGTGCCTGAATG
Sufl17L18L19L20L-F	GCATCGGGGATTGCACTTCTGCTGCTGCTGGTTCCCCTG AAGGCCAGC
Sufl17L18L19L20L-R	GCTGGCCTTCAGGGGAACCAGCAGCAGCAGAAGTGCAAT CCCCGATGC
SuflF	ATGTCACTCAGTCGGCGTC
SuflR	TTACGGTACCGGATTGACC
SuflF8X1	CAGTCGGCGTCAGNNNATTCAGGCATCGG
SuflF8X2	CCGATGCCTGAATNNNCTGACGCCGACTG
FAT75TatChis-1	GAAAGCGAAAAAACTGAAGAACATCACCATCACCATCACT AAGGGCCCCATTAAAGAG
FAT75TatChis-2	CTCTTTAATGGGGCCCTTAGTGATGGTGATGGTGATGTTC TTCAGTTTTTTCGCTTTC
FAT75SuflFLAG-1	GTTGGTCAATCCGGTACCGGATTACAAGGATGACGACGA TAAGTAAGCTTAATTAGCTGAGCTTG
FAT75SuflFLAG-2	CAAGCTCAGCTAATTAAGCTTACTTATCGTCGTCATCCTT GTAATCCGGTACCGGATTGACCAAC
DsbAss-FE	CCGGAATTCGTTTTACATGGAGCAAATATGAAAAAGATTT GGCTGGCG
DsbAss-R	GTTCGTCTTTCGCCGATGCGCTAAACGC
DsbAss16inK-R	GTTCGTCTTTCGCCGATGCGCTtttAAACGCTAAAACTAAAC
DsbA-mAmiA-F	AGCGCATCGGCGAAAGACGAACTTTTAAAAACC
OmpA-FE	CCGGAATTCGTTTTACATGGAGCAAATATGAAAAAGACAG CTATCGCG
OmpAss-R	GTTCGTCTTTGGCCTGCGCTACGGTAG
OmpA18inK-R	GTTCGTCTTTGGCCTGCGCTACTTTGGTAGCGAAACCAG
OmpA-mAmiA-F	GTAGCGCAGGCCAAAGACGAACTTTTAAAAACC
amiA-mRX	GAC TCT AGA TTA TCG CTT TTT C
FATHF-DsbA-F	CACCATCACTAAGGGCCCCATTAAAGAGGAGAAATTAACC ATGAAAAAGATTTGGCTGGCG
FATHF-OmpA-F	CACCATCACTAAGGGCCCCATTAAAGAGGAGAAATTAACC ATGAAAAAGACAGCTATCGCG
FATHF-OmpA18K-R	CAAGTAGCGGCGGAACGGGTAGCGGTTGCTGTTGCCCG GCGGCCTGCGCTACTTTGGTAG
OmpAFLAG-SR	ACGCGTCGACTTACTTATCGTCGTCATCCTTGTAATCAGC CTGCGGCTGAGTTAC

DsbAFLAG-SR	ACGCGTCGACTTACTTATCGTCGTCATCCTTGTAATCTTTT TTCTCGGACAGATATTTCAC
QEF	CCCGAAAAGTGCCACCTG

Table S3. Oligonucleotides used in this study.

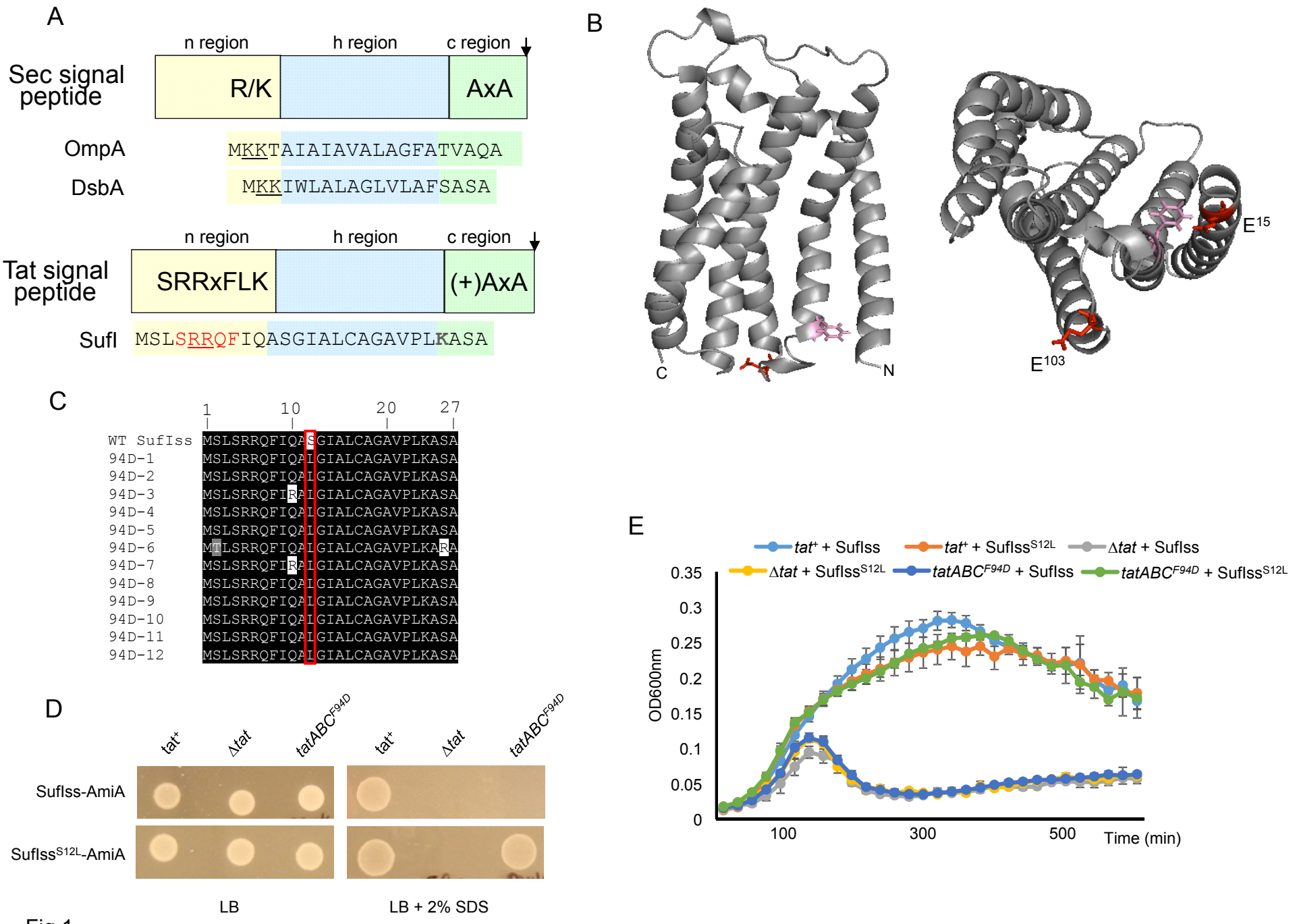


Fig 1

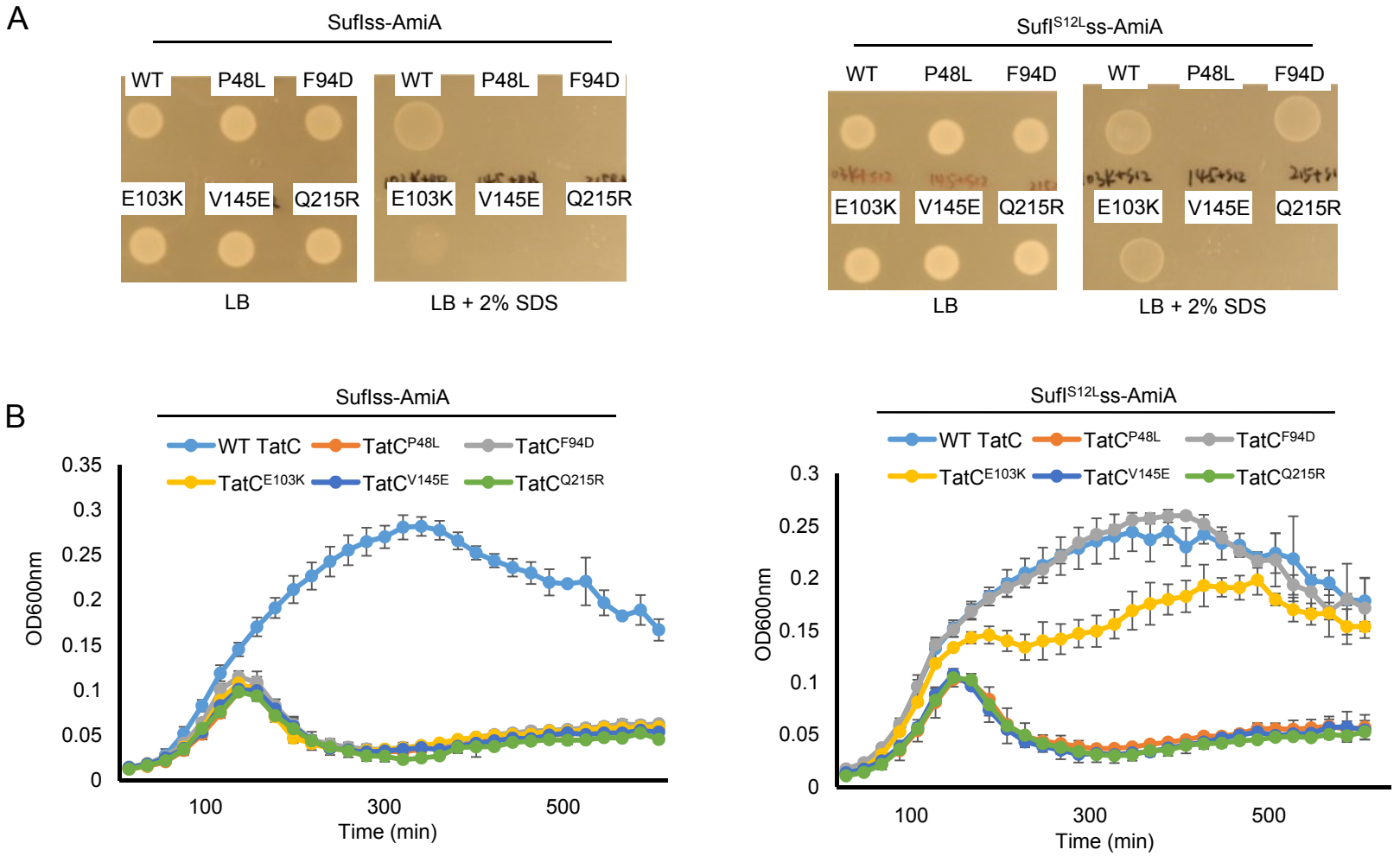
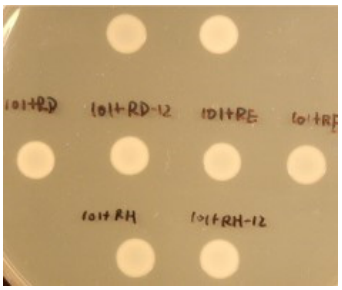
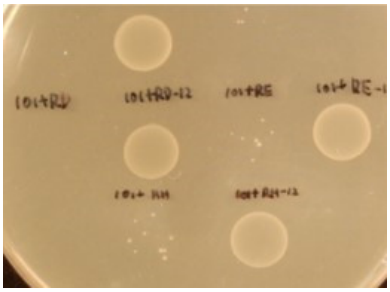


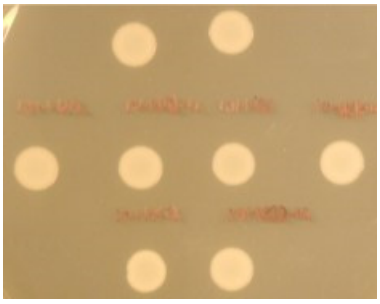
Fig 2

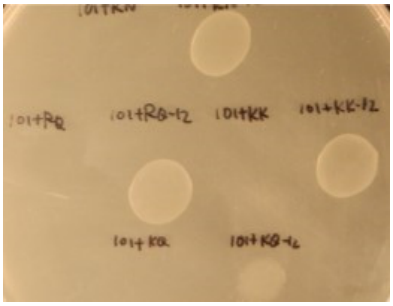
A





	tat⁺	Δ*tat*	
R6D	R6D S12L	R6E	R6E S12L
	RH	R6H S12L	

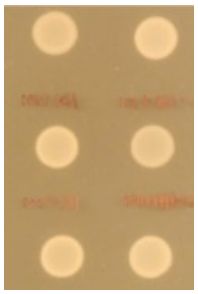


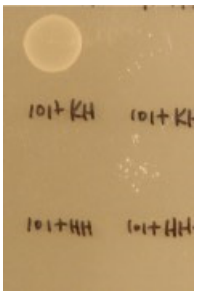


	R6N	R6N S12L	
RQ	RQ S12L	R5K R6K	R5K R6K S12L
	R5K R6Q	R5K R6Q S12L	

LB LB + 2% SDS

B

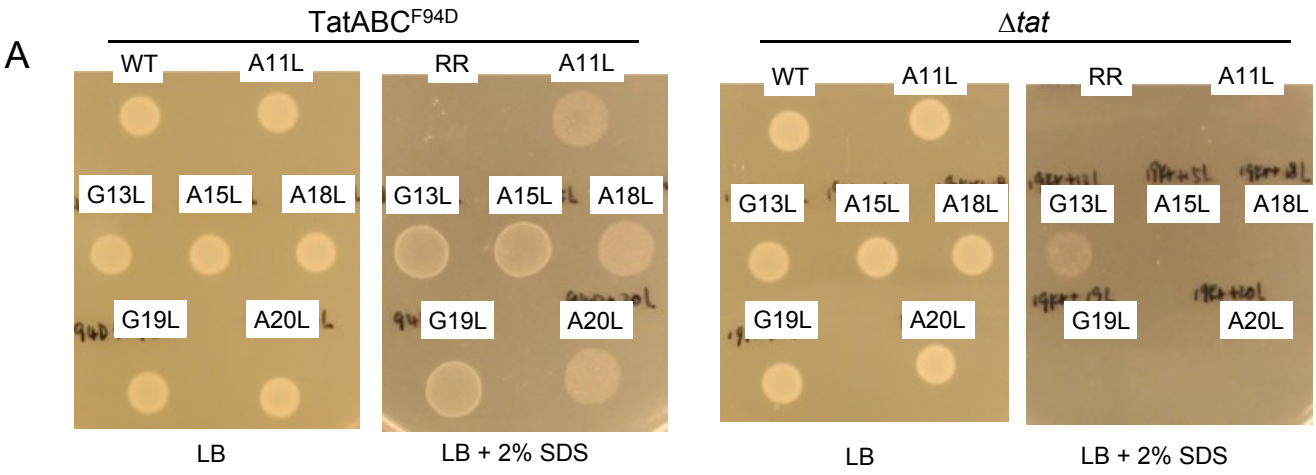


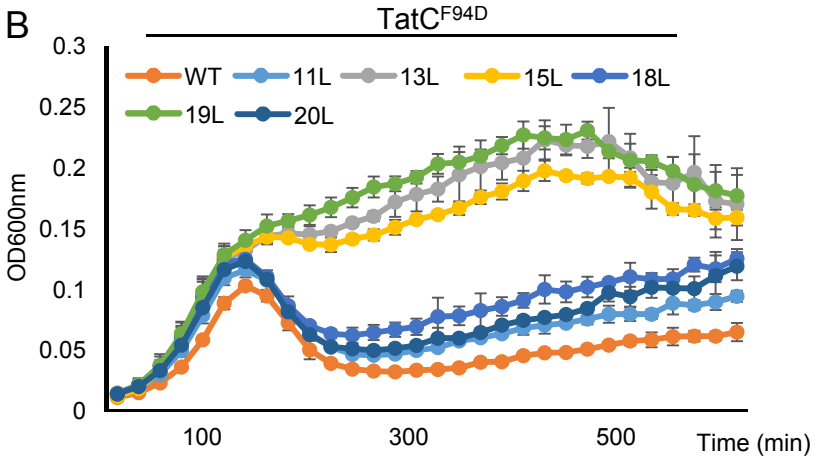


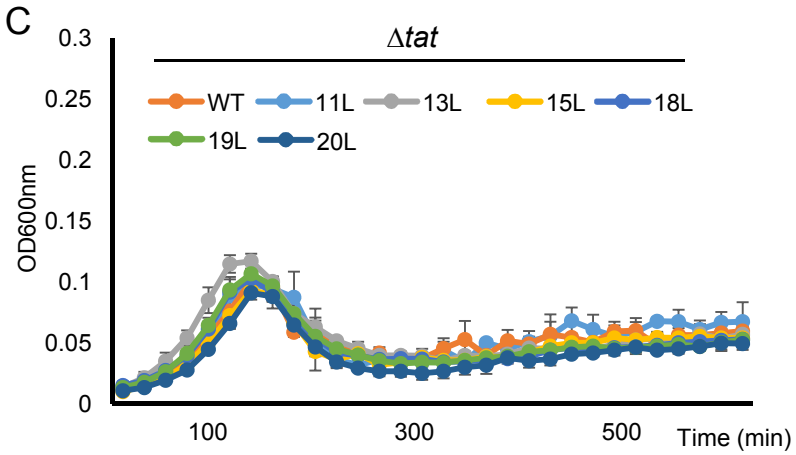
tat⁺	Δ*tat*
R5K R6H	R5K R6H S12L
R5H R6H	R5H R6H S12L

LB LB + 2% SDS

Fig 3







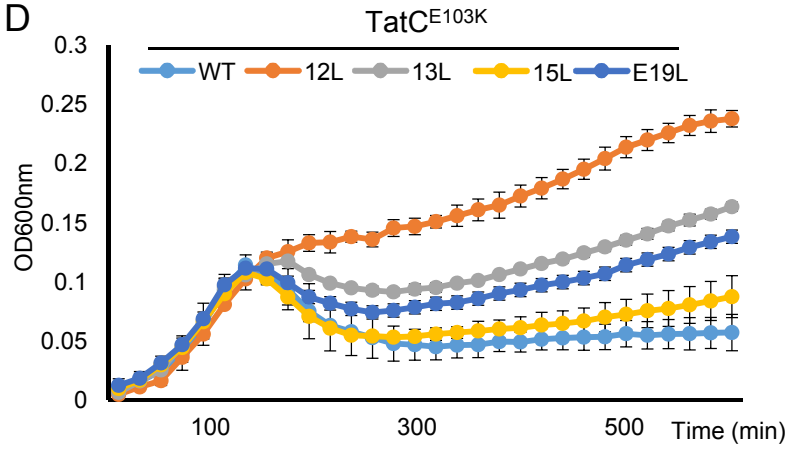


Fig 4

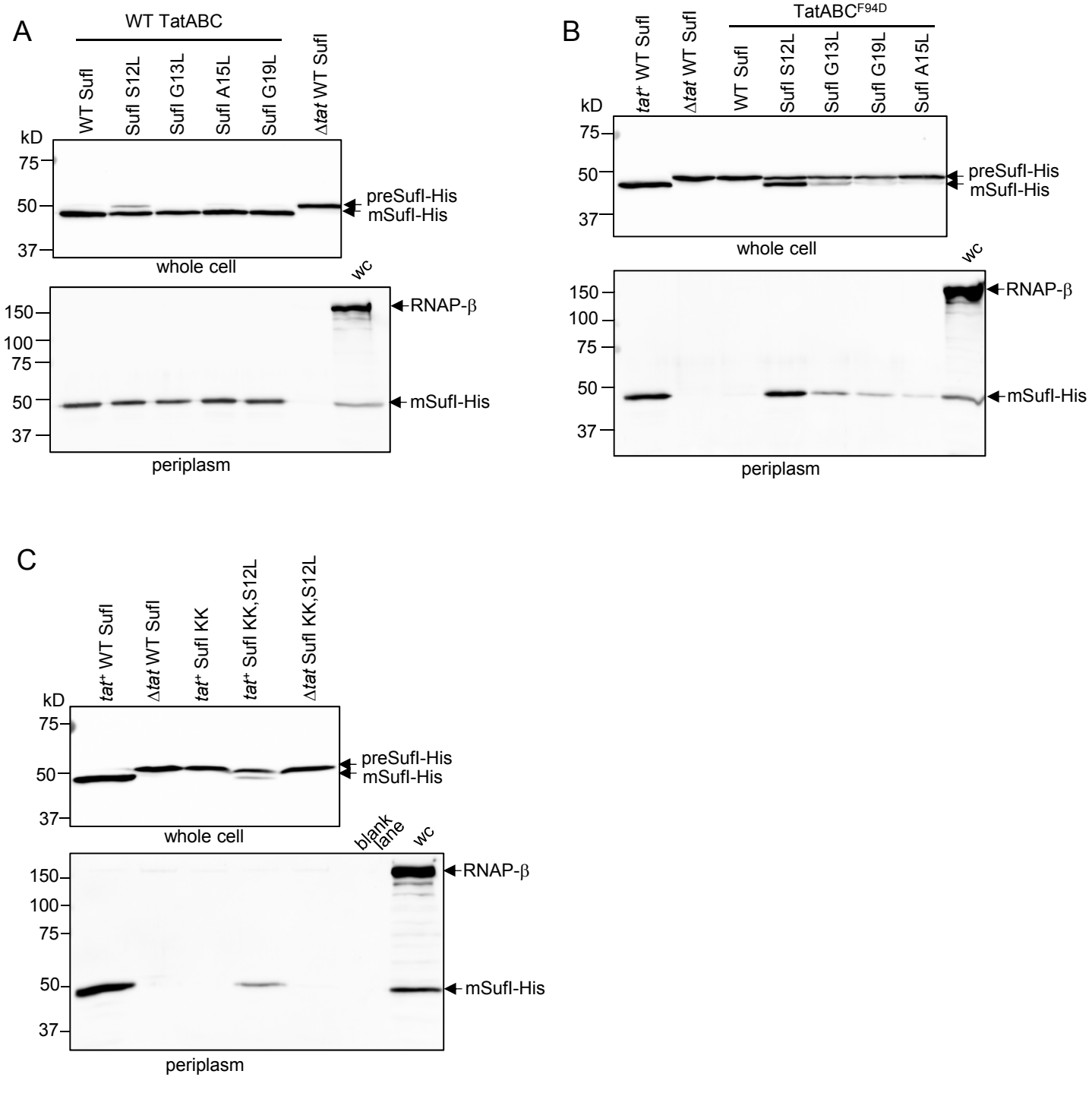
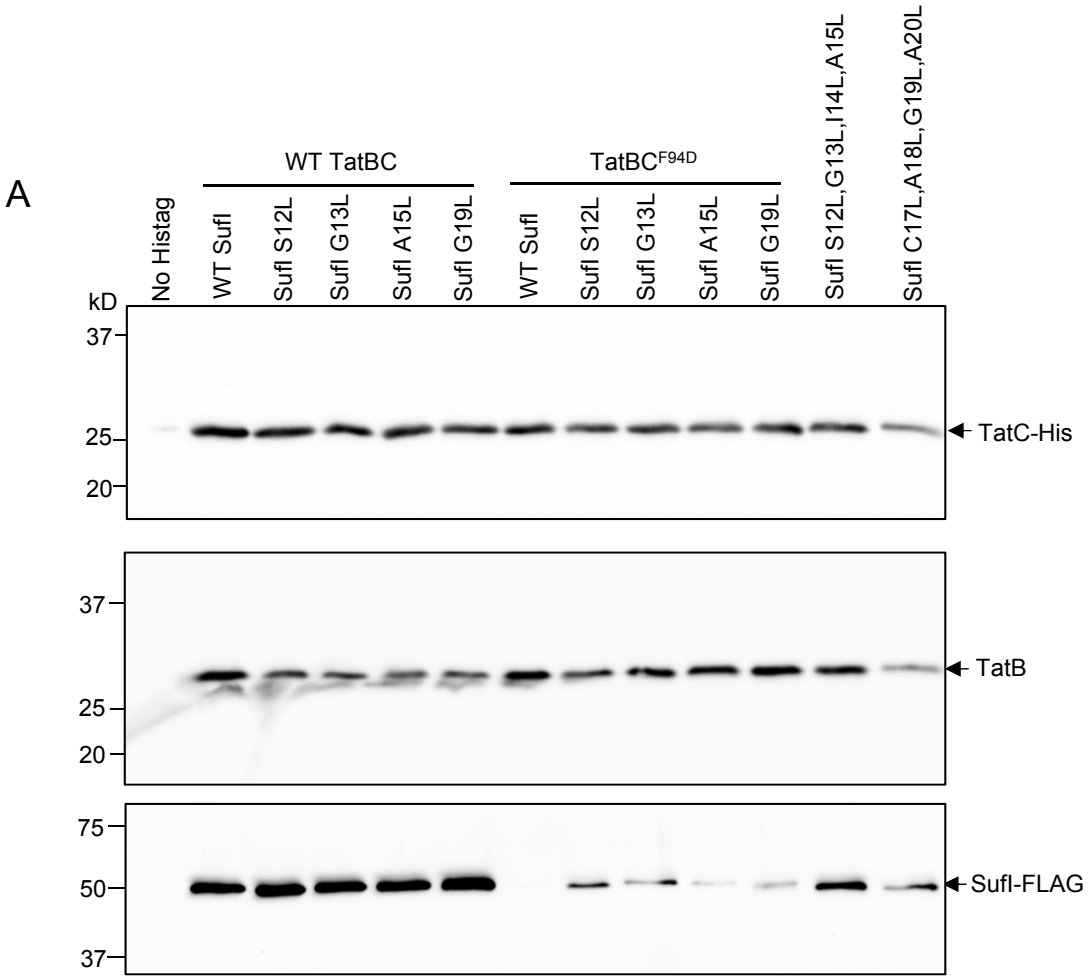


Fig 5



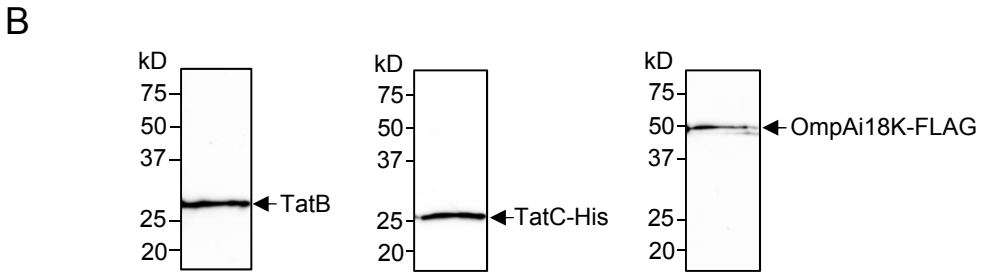
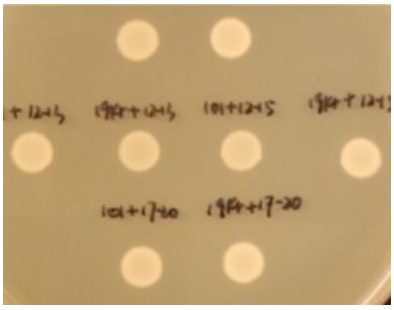
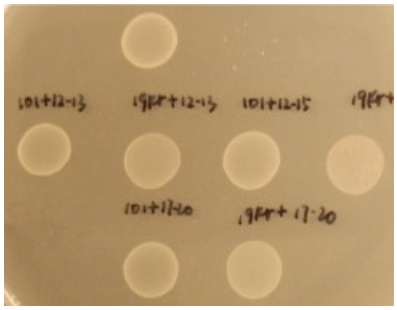


Fig 6

A



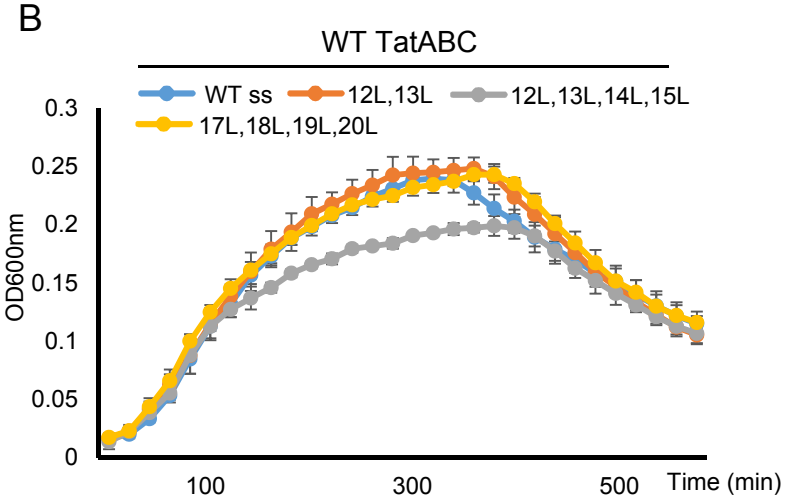


	tat⁺ WT SufIss	*tat*⁻ WT SufIss	
tat⁺ 12L, 13L	*tat*⁻ 12L, 13L	*tat*⁺ 12L, 13L, 14L, 15L	*tat*⁻ 12L, 13L, 14L, 15L
	tat⁺ 17L, 18L, 19L, 20L	*tat*⁻ 17L, 18L, 19L, 20L	

LB

LB + 2% SDS

B



C

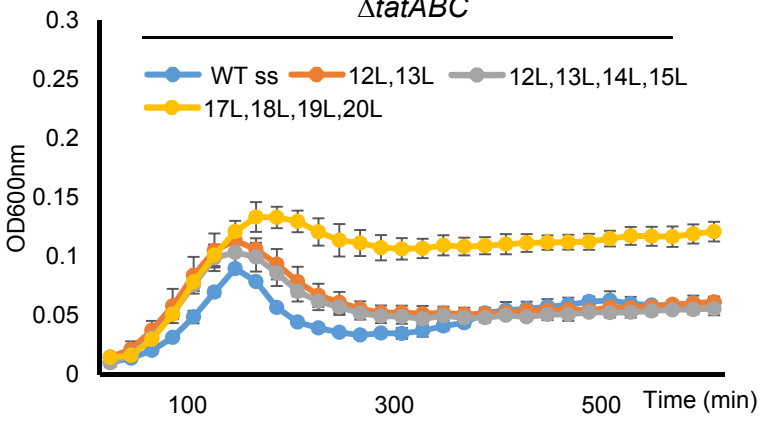


Fig 7

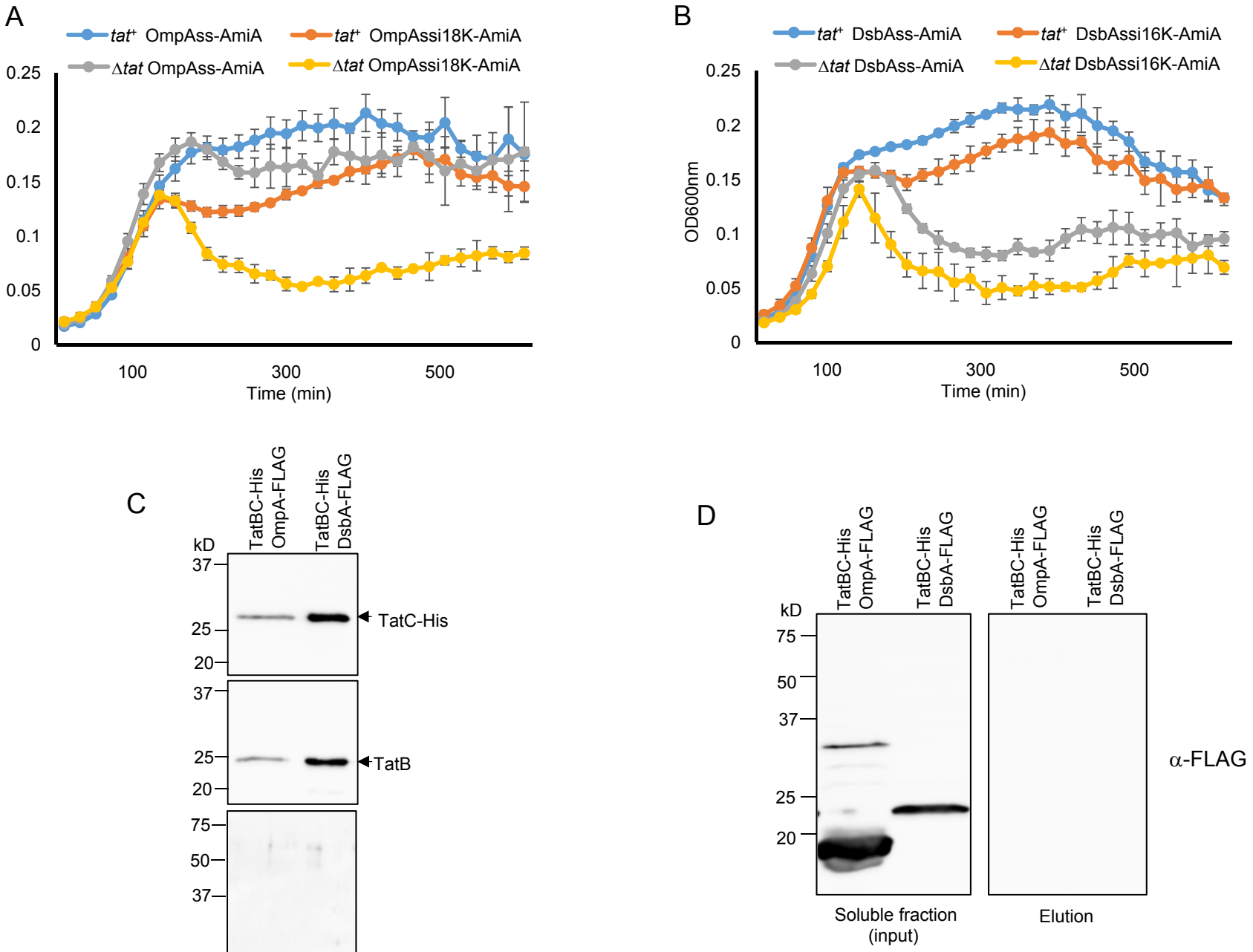


Fig 8

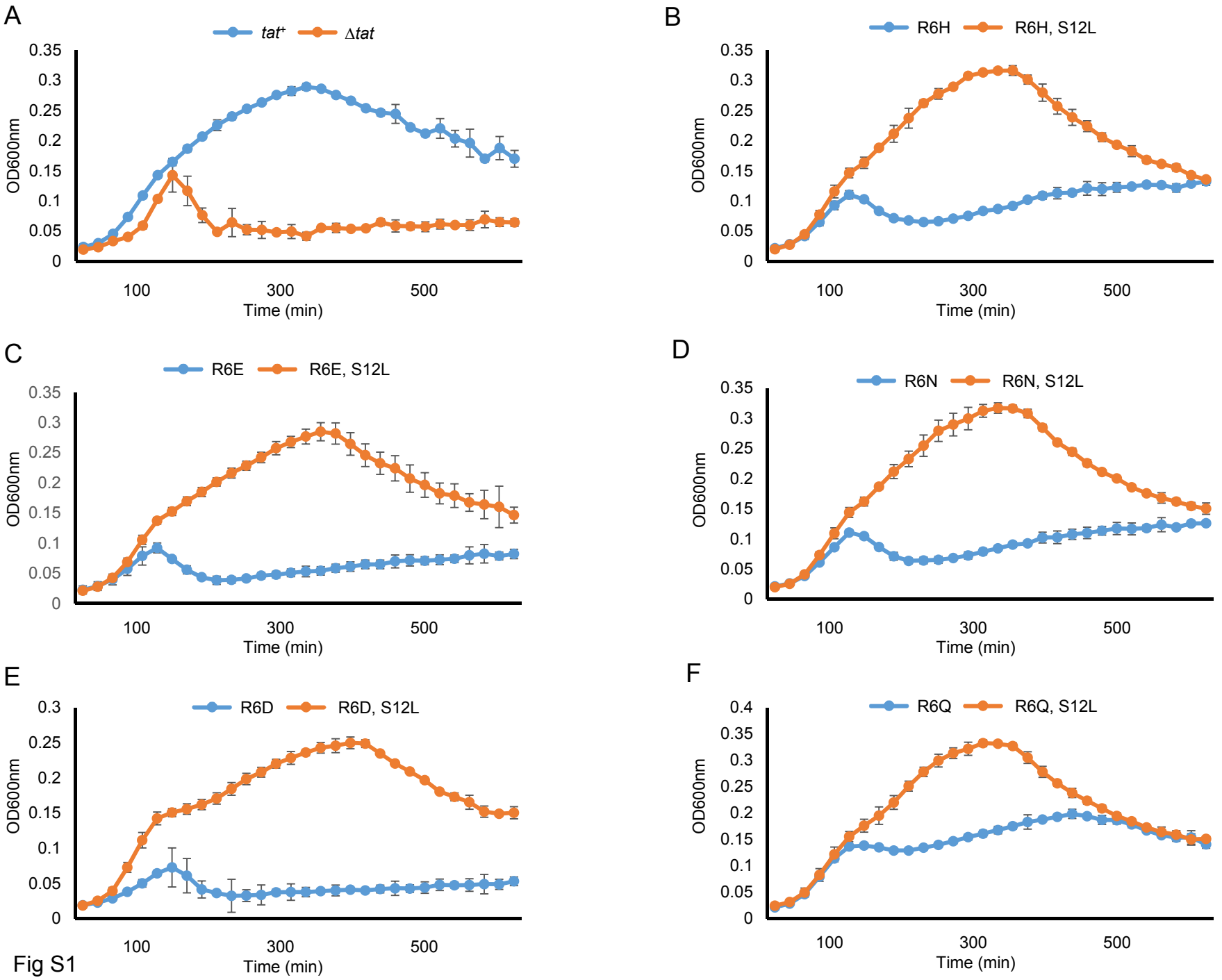


Fig S1

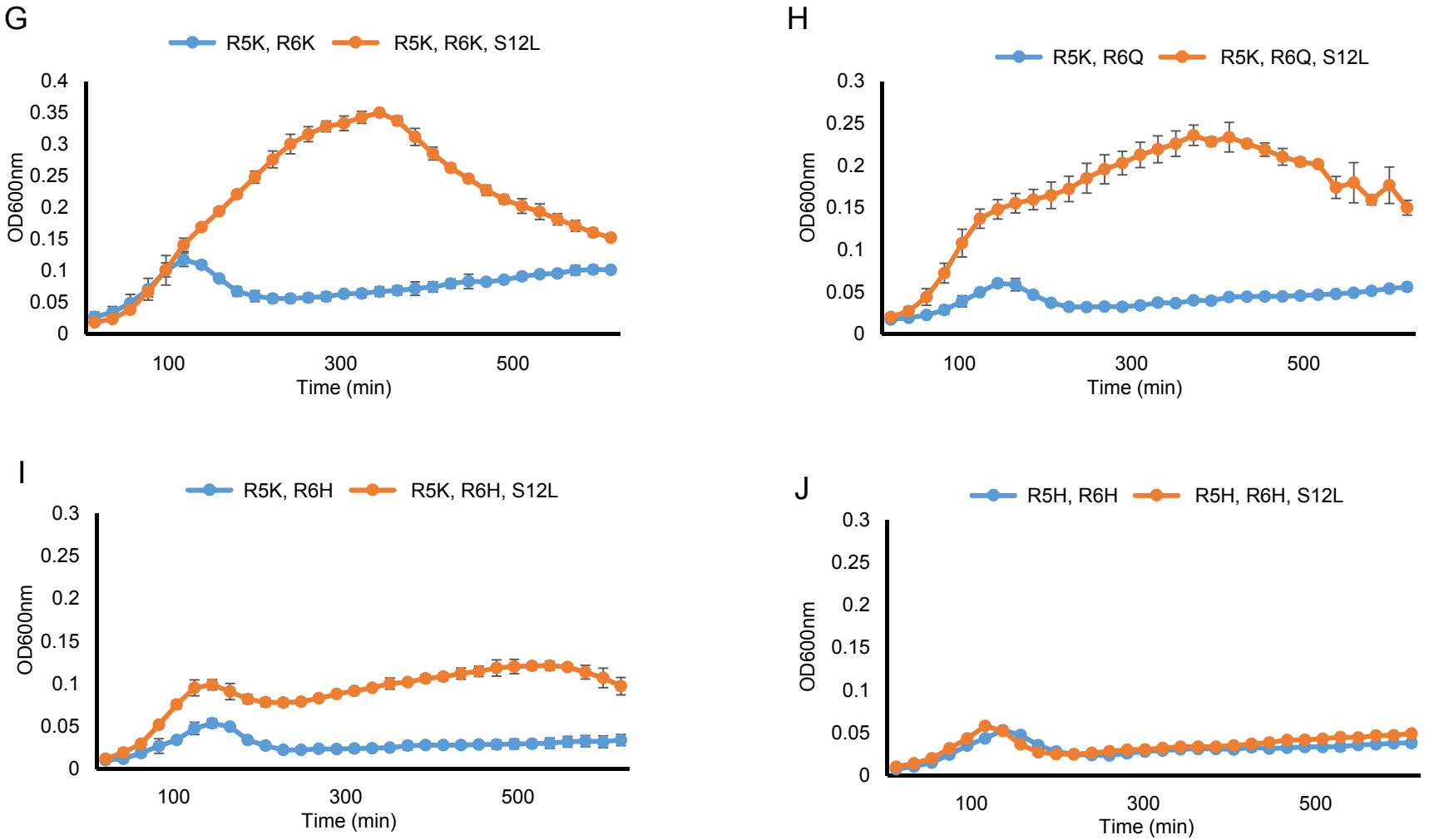
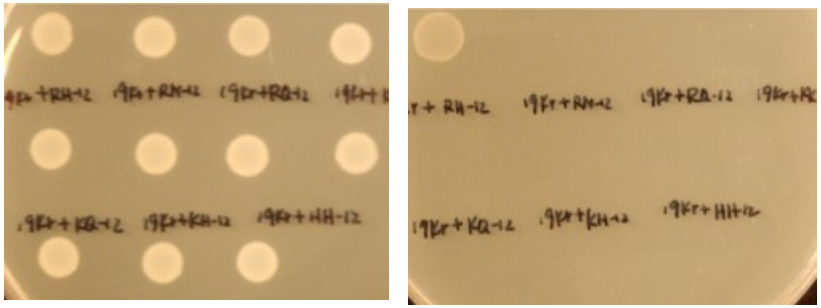


Fig S1…continued

A



tat⁺	Δ*tat*	R6D S12L	R6E S12L
R6H S12L	R6N S12L	R6Q S12L	R5K R6K S12L
R5K R6Q S12L	R5K R6H S12L	R5H R6H S12L	

B

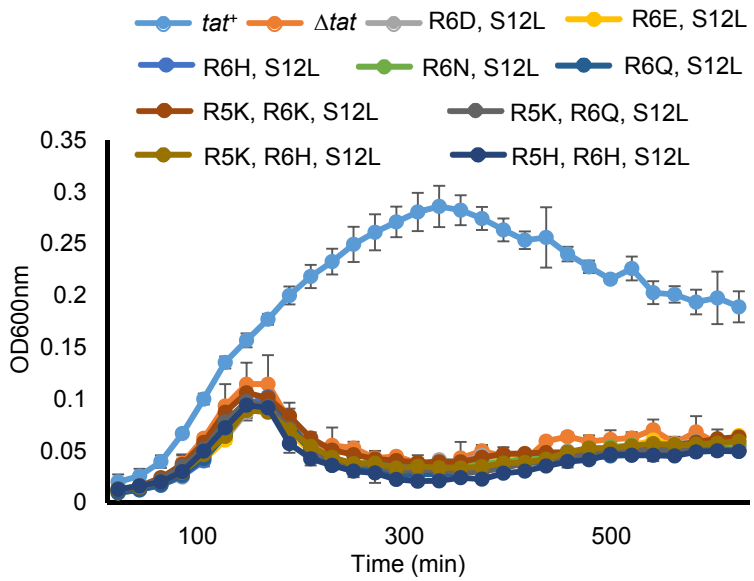
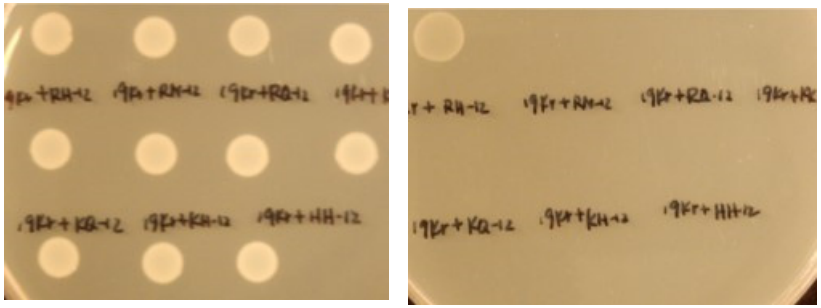


Fig S2

A



tat⁺	Δ*tat*	R6D S12L	R6E S12L
R6H S12L	R6N S12L	R6Q S12L	R5K R6K S12L
R5K R6Q S12L	R5K R6H S12L	R5H R6H S12L	

B

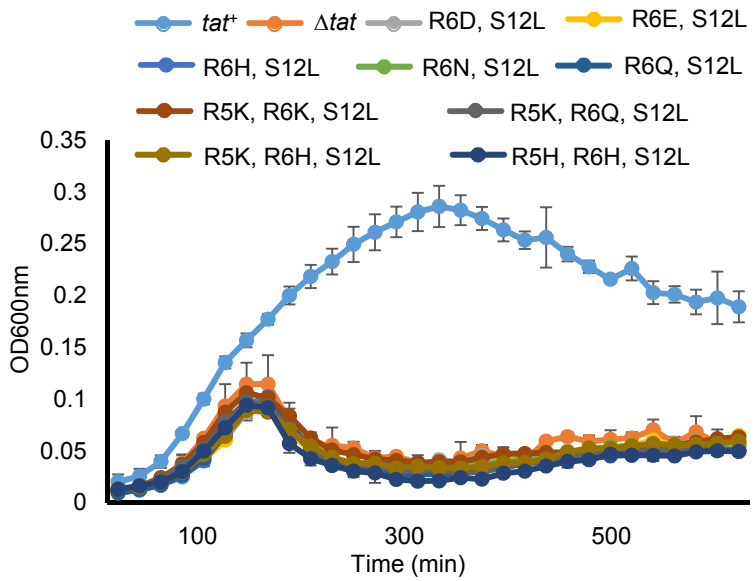
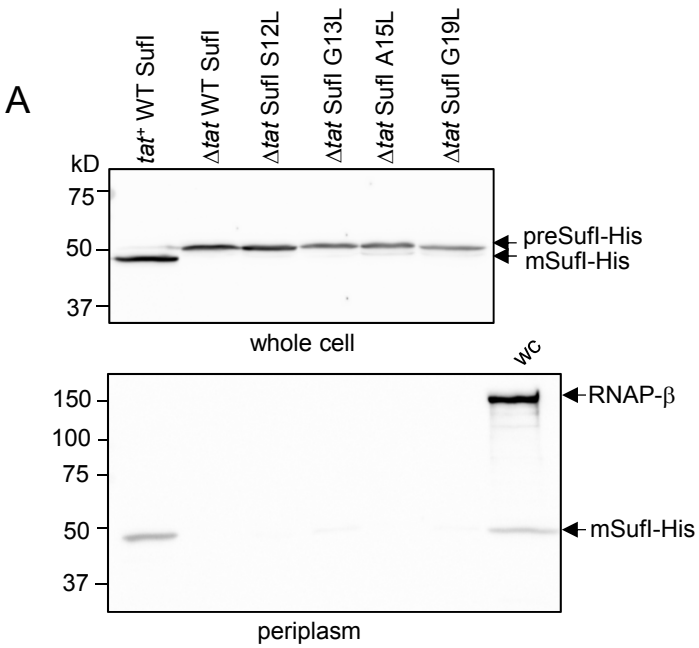


Fig S2



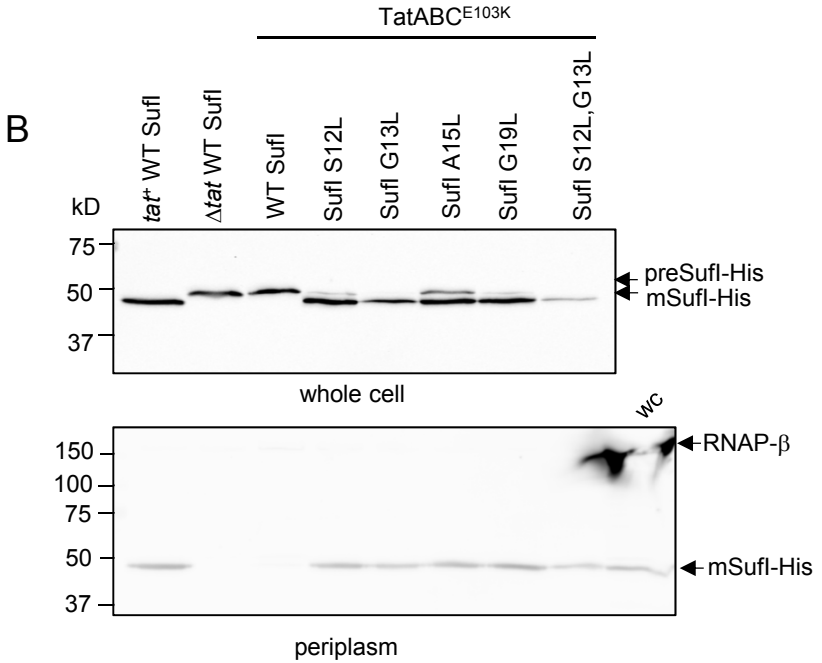


Fig S4

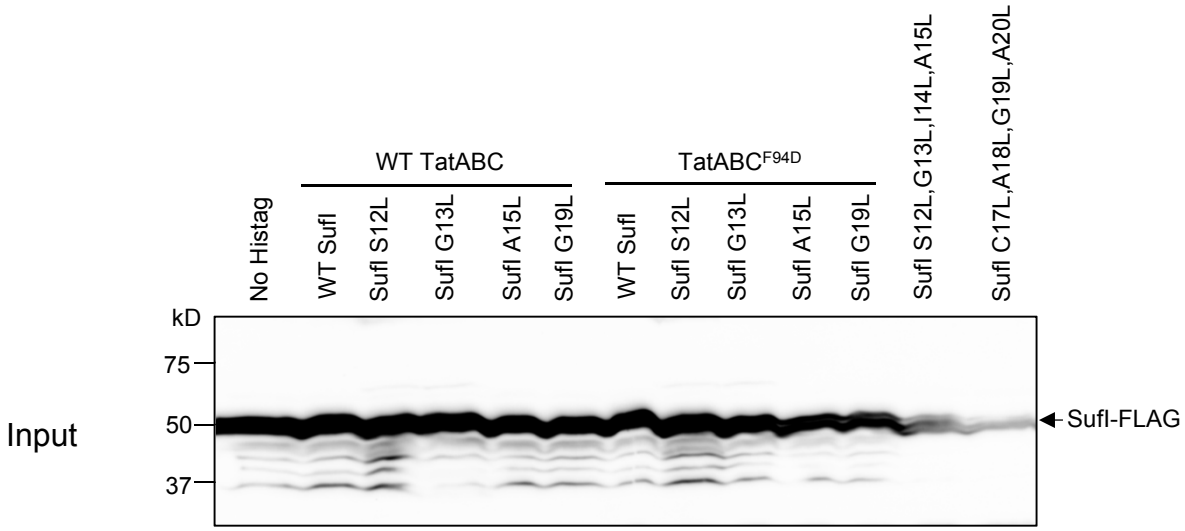


Fig S5