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Published in:
Proceedings of the National Academy of Sciences

DOI:
10.1073/pnas.1615056114

Publication date:
2017

Document Version
Peer reviewed version

Link to publication in Discovery Research Portal

Citation for published version (APA):
A signal sequence suppressor mutant that stabilizes an assembled state of the twin arginine translocase

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Key words: protein transport, twin arginine signal peptide, Tat pathway, genetic suppressor

Abstract
The twin-arginine protein translocation (Tat) system mediates transport of folded proteins across the cytoplasmic membrane of bacteria and the thylakoid membrane of chloroplasts. The Tat system of *Escherichia coli* is made up of TatA, TatB and TatC components. TatBC comprise the substrate receptor complex, and active Tat translocases are formed by the substrate-induced association of TatA oligomers with this receptor. Proteins are targeted to TatBC by signal peptides containing an essential pair of arginine residues. We isolated substitutions, locating to the transmembrane helix of TatB that restored transport activity to Tat signal peptides with inactivating twin arginine substitutions. A subset of these variants also suppressed inactivating substitutions in the signal peptide binding site on TatC. The suppressors did not function by restoring detectable signal peptide binding to the TatBC complex. Instead, site specific crosslinking experiments indicate that the suppressor substitutions induce conformational change in the complex and movement of the TatB subunit. The TatB F13Y substitution was associated with the strongest suppressing activity, even allowing transport of a Tat substrate lacking a signal peptide. *In vivo* analysis using a TatA-YFP fusion showed that the TatB F13Y substitution resulted in signal peptide independent assembly of the Tat translocase. We conclude that Tat signal peptides play roles in substrate targeting and in triggering assembly of the active translocase.

Significance statement
The twin-arginine translocation (Tat) system transports folded proteins across the prokaryotic inner membrane and the thylakoid membrane of plant chloroplasts. Proteins are targeted to the Tat system by signal peptides containing a highly conserved twin arginine motif. We isolated suppressors in the TatB component that allowed a Tat substrate with a defective twin arginine motif to be transported. The strongest of these suppressors, TatBF13Y, resulted in the constitutive assembly of the Tat translocase in the absence of signal peptide binding. These results show that Tat signal peptides have two separable roles – they target their
passenger proteins to the Tat machinery but they also trigger the assembly of the active Tat transporter.
Introduction.

A large proportion of prokaryotic proteins are trafficked into or across the cytoplasmic membrane. Extracytoplasmic proteins are synthesized with cleavable N-terminal signal peptides to address them to export machineries located in the cytoplasmic membrane. Signal peptides are generally between 20-30 amino acids in length and have a recognizable tripartite structure comprising a basic n-region, a hydrophobic h-region and a polar c-region with a signal peptidase cleavage site.

The Sec pathway is the major route of protein export in most prokaryotes, transporting unfolded polypeptides across the cytoplasmic membrane. In bacteria it is comprised of a SecYEG channel complex and a peripheral membrane ATPase, SecA (see (1, 2) for recent reviews). The initial discovery of Sec components was driven by genetic approaches using *Escherichia coli* to isolate suppressors of defective Sec substrate proteins and inactive signal peptides (e.g. 3, 4). These genetic suppressors also contributed significantly to mechanistic understanding of Sec-dependent protein translocation (5-7). More recently it has been shown that Sec signal peptides have dual roles – they serve to target their passenger proteins to the Sec machinery (3, 8) but also to allosterically activate the SecY channel (9).

The twin-arginine protein translocation (Tat) pathway operates in parallel to the Sec pathway to transport folded proteins across the prokaryotic cytoplasmic membrane and the thylakoid membrane of plant chloroplasts (10, 11). Proteins are targeted to the Tat pathway by N-terminal signal peptides that contain a conserved twin arginine motif at the n-region/h-region boundary (12, 13; Fig 1A). The Tat system in the model bacterium *E. coli* requires three membrane-bound subunits, TatA, TatB and TatC (14-17). The TatB and TatC proteins form a multivalent complex that binds Tat substrates through their twin arginine signal peptides (e.g. (18-20). Numerous experiments have shown that the TatC component recognizes the twin arginine motif (21-26) whereas TatB is close to the signal peptide h-region (27, 28). Signal peptides have been shown to penetrate deeply into the TatBC complex (29) and in thylakoids
at least this deep-binding mode may be modulated by the transmembrane proton
electrochemical gradient (PMF) (30).

It is generally accepted that the TatA protein forms the protein-conducting element of the Tat
pathway. TatA oligomers assemble at the substrate-bound TatBC complex, dependent on the
PMF (27, 31-35). Current models for Tat transport propose that TatA oligomers either provide
form-fitting channels of varying diameter that adapt to the size of the folded passenger domain,
or that oligomeric assemblies of TatA cause a localized weakening of the membrane and
transient bilayer disruption accompanied by substrate transport (reviewed in 10, 11). An
implicit prediction of the latter model is that transient membrane rupture would be expected to
be accompanied by ion leakage.

In this study we have addressed the function of the twin-arginine signal peptide in the Tat
transport process by isolating genetic suppressors that either restore transport to signal
peptides harbouring transport inactivating twin arginine substitutions, or that restore Tat
activity to a TatC variant that has an inactive signal sequence binding site. Our results
identified a common set of substitutions, primarily located in the transmembrane helix of TatB
that can suppress both types of transport defect. Biochemical analysis of Tat translocases
harboring these substitutions indicates that at least one of them, TatB F13Y, promotes signal-
peptide independent TatA assembly. Our findings show that, like Sec signal peptides, Tat
targeting sequences also play two roles in the transport process.
Results.

Single amino-acid substitutions in TatB permit export of twin-arginine substituted signal peptides.

Previous genetic screens using maltose binding protein (21, 22) or GFP (23) fused to the Tat signal sequence of E. coli TorA identified mutations that were able to restore some level of Tat transport to fusions with export defective twin arginine substitutions. These substitutions were located towards the N-terminal end of the TatB transmembrane helix or within the cytoplasmic loops of TatC. To shed light on potential functions of signal peptides during Tat transport, we initiated an independent genetic screen using a native E. coli Tat substrate, AmiA, as our reporter. AmiA is an N-acetylmuramoyl-L-alanine amidase that remodels the cell wall during growth. In the absence of a functional Tat system, the cell envelope is impaired due to the inability to correctly localize AmiA and the related Tat substrate AmiC, rendering E. coli sensitive to killing by SDS (36). Utilizing a strain lacking chromosomal tat genes and amiA/amiC (37), co-production of plasmid-encoded AmiA (from the medium copy construct pSUAmiA; 36) alongside a separate plasmid, pTAT1d (producing TatABC from a compatible medium copy plasmid; 38), permits the strain to grow on LB medium containing 2% SDS (SI Appendix, Fig S1). We then mutated the consecutive arginines in the Tat signal peptide of plasmid-encoded AmiA to each of the amino acid pairs RD, RE, RH, RN, RQ, KH, KQ or HH. As expected, each of these substitutions abolished growth on SDS (SI Appendix, Fig S1).

Next we screened a tatB mutant library, generated in pTAT1d by error-prone PCR, for clones that supported growth of strains carrying AmiA with variant signal peptides (38). Each AmiA twin arginine variant was challenged with this library, screening approximately 10,000 clones for each construct. In total across the screening campaign we isolated thirty individual clones. Upon re-screening twenty of these retained the ability to suppress the inactive signal peptide variant of AmiA against which they were originally isolated. These clones are listed in SI Appendix, Table S1. Substitutions appeared to cluster within the transmembrane region of TatB, including L9Q that appeared in seven of the clones and F13Y that occurred in five clones, whilst F6Y, E8K, L9P and L10P were each found once. It should be noted that E8K,
L9P and L9Q substitutions have previously been identified as suppressors of inactive signal peptides (21, 22). We also found clones, each isolated twice, that contained no substitutions in the transmembrane domain, where the first substitution was in the amphipathic helix of TatB (clones BRQ1, BRQ2, BRQ3 and BRQ5).

We introduced the individual amino acid substitutions F6Y, E8K, L9P, L9Q, L10P, F13Y, K30I and I36N into TatB encoded within the tatABC operon on the very low copy number plasmid pTAT101 (39) and tested their ability to support export of wild type AmiA and to suppress each of the RD, RE, RH, RN, RQ, KH, KQ or HH AmiA signal sequence variants (Fig 1B, 1C, SI Appendix, Fig S2, Table 1). Each of the substitutions was able to support transport of wild type AmiA, but they varied in their ability to permit export of the AmiA signal sequence variants. For example the F13Y variant of TatB supported growth on SDS for all of the signal peptide variants tested, whereas the L9Q, L10P, K30I and I36N TatB substitutions could suppress some of the signal sequence variants but not KH, KQ and HH substitutions. To determine whether the suppression observed was specific for the AmiA signal peptide, we generated a further construct where the signal peptide of the Tat substrate SufI was fused to the mature portion of AmiA and the same twin arginine substitutions were introduced (SI Appendix, Fig S3, Table 1). The TatB variants generally showed the same pattern of suppression of SufI signal peptide substitutions, although the TatBL10P, K30I and I36N variants could not suppress RD or RE substitutions in the SufI signal peptide.

A subset of TatB signal peptide suppressors also suppress TatC signal peptide binding site mutants.

In a complementary approach we asked whether it was possible to select suppressors of defects in the signal peptide binding site on TatC. Residue F94 in E. coli TatC is highly conserved and lies within the signal peptide binding site (25, 40; Fig 2A), and substitution to other amino acids is poorly tolerated (41). We constructed substitutions of F94 to each of the small neutral amino acids Ala and Gly, helix breaking Pro, polar residues Ser and Gln, positively charged Arg and Lys and negatively charged Asp. These substitutions were
introduced into TatC encoded on both the medium copy plasmid pTAT1d and the very low

161 copy plasmid pTAT101 that also carry wild type tatA and tatB. SI Appendix, Fig S4 shows that

162 substitutions to Asp, Gln or Pro resulted in a complete inability of strain DADE (ΔtatABCD,

163 ΔtatE; 42) to grow in the presence of SDS at both medium and very low copy number. We

164 selected the F94Q substitution of TatC and constructed three mutant libraries in the pTAT1d

165 vector by error prone PCR in an attempt to identify suppressors of this inactivating tatC

166 mutation. LibC1 carried mutations in the first 93 codons of tatC, LibC2 carried mutations in

167 tatC from residue 95 onwards and LibAB contained mutations in the tatA and tatB genes.

A number of suppressors were identified from screening the LibC1 and LibC2 libraries for
growth on SDS plates. However sequence analysis indicated that for each of these Tat active

171 mutants there was substitution at the tatC F94Q codon to tatC F94Y, W or L codons. By

172 contrast, after screening more than 180 000 clones from the LibAB library, eleven mutants

173 were isolated which were able to rescue the growth defect of TatC F94Q on SDS plates, of

174 which five were still able to support growth on SDS following fresh transformation of strain

175 DADE with the isolated plasmid. These clones are listed in SI Appendix, Table S2.

Interestingly, each of these suppressors encoded either TatB L10P, F13Y or I36N that had

177 been identified in our prior screen for signal sequence suppressors. Introduction of each of

178 these substitutions, individually, into the very low copy number pTAT101CF94Q plasmid

179 supported growth of strain DADE on SDS-containing media (Fig 2B), indicating that these

180 TatB variants are each able to rescue the Tat-inactivating F94Q TatC substitution.

Since these three TatB substitutions that suppress the TatC F94Q defect were previously

181 isolated as suppressors of inactive Tat signal sequences, we asked whether any of the other

182 tatB signal sequence suppressors we had found could also rescue the TatC F94Q substitution.

Fig 2C shows that in addition to L10P, F13Y and I36N, L9Q could also restore Tat activity to

184 cells producing TatC F94Q. The location of each these residues on a model of TatB is shown

185 in Fig 2D. We next asked the question whether any of these TatC F94Q suppressors could

186 restore Tat activity to other inactivating substitutions in TatC. Fig 2E shows that two different
inactivating substitutions of E103 in the signal peptide binding site, either E103A or E103K (25, 40, 41; Fig 2A), could also be complemented by three of the four suppressors of tatCF94Q (I36N did not suppress these substitutions), but they could not restore Tat activity caused by inactivating TatC substitutions located outside the signal peptide binding site (SI Appendix, Fig S5). Finally we tested whether F94Q suppressing substitutions were additive, i.e. whether when combined they resulted in a stronger suppressing activity. However, SI Appendix, Fig S6A shows that none of the pairwise combinations we tested gave any suppression of tatCF94Q and, with the exception of the F13Y, I36N substitution which showed some suppression of the RN signal peptide variant, the combined suppressors lost suppressive function of RN or KK substitutions of the SufI signal peptide (SI Appendix, Fig S6B). We therefore conclude that the suppressor mutations, when combined, have detrimental rather than additive effects on suppression activity.

**The TatB F13Y and L9Q substitutions support export of AmiA lacking a signal peptide.**

The results above indicate that the TatBF13Y substitution is the strongest suppressor of inactive Tat signal peptides, allowing the *E. coli* Tat system to recognize all eight of the different twin arginine motif substitutions tested as well as suppressing the TatC F94Q mutation. We therefore tested whether more severe signal peptide defects could be suppressed by this TatB variant. Fig 2F shows that, remarkably, even after truncation of the signal peptide by removal of the h-region, or indeed complete removal of the entire signal peptide-coding sequence of AmiA, we could still detect some Tat-dependent translocation of the AmiA passenger domain in the presence of TatBF13Y. The TatB L9Q substitution, which was the strongest suppressor of signal peptide defects after F13Y, also supported some translocation of mature AmiA, however we were not able to detect export of mature AmiC in the presence of either of these two suppressor substitutions (SI Appendix, Fig S6C). We conclude that TatB L9Q and F13Y allow at least one Tat substrate to be transported independent of any signal peptide.
Variant TatB proteins support good transport activity of a native Tat substrate but much poorer transport when the signal peptide is altered.

We next addressed whether the TatB substitutions were detrimental to the activity of the Tat system. Using overproduced his-tagged, but otherwise native SufI as a substrate it was seen that mature SufI was clearly detected in the periplasmic fractions of all of the strains tested, although TatBI36N seemed to support only low levels of transport (SI Appendix, Fig S7A). However, we were unable to detect transport of the RD, RN or KQ signal peptide variants of SufI in the presence of TatBF13Y (SI Appendix, Fig S7B) or of a twin-lysine substituted his-tagged CueO in the presence of TatBE8K or F13Y (Fig 3). It therefore appears that there is very low export efficiency of substrates with variant signal peptides in the suppressor mutant strains.

The TatB suppressors do not restore biochemically detectable signal peptide binding to TatBC.

We subsequently sought to understand the biochemical basis for the action of the TatB suppressors. Our initial hypothesis was that they acted to increase the affinity of the TatBC complex for the variant signal peptides, or to restore binding to complexes containing the TatC F94Q or E103A/E103K substitutions. First we produced his-tagged GFP with variants of the SufI signal peptide at its N-terminus and assessed how much TatBC could be co-purified with this from detergent-solubilized membrane fractions. Fig 4A shows that when the wild type signal peptide was fused to GFP, wild type TatBC or variants harbouring the TatB E8K, F13Y or I36N substitutions were co-eluted with his-tagged SufI-GFP. However no TatBC was detected when the RR motif in the signal peptide was mutated to RD, RN, or KK, even in the presence of the TatB suppressor substitutions (despite the fact TatBC and GFP were clearly present in all of the input samples; SI Appendix, Fig S8A and B). Very similar behavior was also seen when his-tagged AmiA variants were used as substrate for co-purification experiments (SI Appendix, Fig S9). Thus TatBC and TatBF13YTatC co-purified with the his-tagged wild type AmiA precursor, but no TatBC was detected when RD, RN, KK or KQ
substitutions were introduced into the signal peptide, or when the AmiA signal peptide was lacking. We conclude that the TatB suppressors do not detectably restore binding of variant signal peptides to the TatBC complex. Since several TatB variants can transport substrates with defective signal peptides, but not without signal peptides (Fig 3), we infer that the defective signal peptides must still weakly interact with the TatBC complex at a level that is not detected by our co-purification assay.

We then tested whether any of the four suppressors, TatB L9Q, L10P, F13Y and I36N, that allow Tat transport in the presence of the TatC F94Q and E103K mutations, acted to restore substrate binding to TatBC complexes containing these signal sequence binding site substitutions. Although high GFP fluorescence and strong TatBC signals were detected in whole cells (SI Appendix, Fig S8C and D), only wild type TatBC was found to co-purify with his-tagged SufIiss-GFP (Fig 4B). Thus, as expected, TatC substitutions F94Q or E103K prevented co-purification of TatBC-substrate complex, consistent with loss of signal peptide binding detected for substitutions at these amino acid positions (25, 40). However, detectable signal peptide binding was not restored by introduction of the individual TatB suppressor substitutions. We conclude that the TatB suppressors do not act by rescuing signal peptide binding.

The TatBC complexes harboring TatB suppressor substitutions are conformationally altered.

We next investigated whether TatBC complexes could still be detected when any of the TatBL9Q, L10P, F13Y or I36N substitutions were present in TatB. Membranes harboring wild type TatA and TatC along with each of these TatB variants were solubilized with digitonin and analysed by blue-native gel electrophoresis (BN-PAGE). As shown in Fig 5A, the wild type TatBC complex solubilized with digitonin migrated close to the 440 kD marker, as reported previously (e.g. 43). The TatB L9Q, F13Y and I36N variants were also associated with a complex of apparently identical size to wild type TatBC, whereas for membranes producing TatBL10P, very little TatBC complex could be detected, even though both proteins were
solubilized from the membrane (SI Appendix, Fig S10). Interestingly, the L9Q and F13Y TatB substitutions also resulted in the appearance of a second band of apparently higher mass that was absent from the sample containing wild type TatBC (Fig 5A, B).

We wondered whether this additional band might arise due to the presence of excess TatA bound to the variant complexes. However, blotting the BN gels for TatA showed the distinct TatA-laddering pattern reported previously (44, 45) was detectable for all of the samples, but there was no obvious TatA cross-reactive material migrating at the same position as the higher mass TatBC-containing complex (Fig 5B). To examine whether the presence of TatA was required for these higher molecular weight variant TatBC complexes to form, we repeated the BN-PAGE analysis in the absence of TatA (or its paralog TatE; 16). Surprisingly, this resulted in the apparent aggregation of the variant TatBC complexes, yielding a series of bands of apparent masses well above 440kDa that were not seen for the wild type (Fig 5C). We infer from this that there is a conformational alteration in the TatBC complex induced by the presence of the L9Q or F13Y TatB substitutions that in the absence of TatA causes further oligomerisation.

Conformational alterations in the TatBC complex have been previously detected by disulfide crosslinking (46, 47). Cléon et al. (47) reported that when a Tat substrate was overproduced, a disulfide crosslink between M205C in transmembrane helix 5 of neighboring TatC proteins could be detected in vivo, suggesting the formation of a transient TatC dimer in response to substrate binding. Fig 5D confirms that dimerization through TatC M205C is not observed unless cells also harbor an overproduced Tat substrate, in this case CueO. The TatC M205C dimer induced by CueO is almost completely absent when the F94Q substitution is introduced into TatC, again supporting the conclusion that substrate binding promotes TatC dimerization (Fig 5D). Interestingly, however, when either the TatB L10P or F13Y substitutions were present, a TatC M205C crosslink was detected in the absence of overexpressed substrate. We wondered whether these TatB substitutions rendered the TatBC complex more responsive to the presence of endogenous substrates. To test this, we also introduced the signal peptide binding defective F94Q substitution into TatC M205C. However, as Fig 5D shows, the TatC
M205C dimer can still be detected in the presence of TatB L10P or F13Y substitutions, even when the F94Q inactivating substitution is present, and is therefore independent of signal peptide binding. We conclude that at least a subset of the TatB suppressors induce conformational changes in the TatBC complex, and that the TatBL10P and F13Y substitutions potentially mimic the substrate-bound form of the complex.

The TatBF13Y substitution promotes signal peptide-independent oligomerisation of TatA in vivo.

Substrate binding to the TatBC complex is a pre-requisite for the assembly of a TatA oligomer. TatA oligomer assembly in vivo can be followed by fluorescence microscopy in cells producing a chromosomally-encoded TatA-YFP fusion protein (34). When Tat substrates are present at native level, TatA oligomers are found with low frequency, but this frequency can be significantly increased by overproduction of a Tat substrate protein with a functional signal peptide (34, 35). This finding is confirmed in Fig 6A, where clusters of TatA-YFP can be seen in cells overproducing AmiA from a plasmid. As expected, introduction of the F94Q codon substitution into chromosomally-encoded tatC prevented the AmiA-induced clustering of TatA-YFP resulting in a halo of delocalized TatA around the cell periphery (Fig 6A), consistent with the inability of the TatC variant to bind substrates. We next assessed whether any of the TatC F94Q suppressors, TatB L9Q, L10P, F13Y or I36N (introduced into chromosomal tatB) affected the oligomerisation of TatA-YFP (Fig 6B, SI Appendix, Fig S11). Remarkably we found that the presence of the TatB F13Y substitution promoted constitutive assembly of TatA-YFP in the absence of overproduced Tat substrates (Fig 6B), and the TatA-YFP assemblies persisted even in the presence of the TatC F94Q substitution for this variant (but not for L9Q, L10P or I36N; SI Appendix, Fig S11). Taken together, these results indicate that the TatB F13Y substitution triggers signal peptide-independent assembly of TatA oligomers.

No leak across the cytoplasmic membrane when cells produce the Tat system containing TatB F13Y.
One of the current models for Tat transport posits that TatA oligomers facilitate transport of substrates by causing a localized weakening of the bilayer and transient disruption (discussed in 10, 11). Such a mechanism might be expected to be accompanied by increased permeability of small molecules associated with assembled TatA. The availability of a TatB variant (F13Y) that causes TatA to accumulate in the assembled state provides an experimental tool to investigate this issue.

First we asked whether overexpression of Tat systems containing the TatB suppressors L9Q, L10P, F13Y or I36N from an arabinose-inducible promoter had any effect on the growth rate of E. coli. Fig 7A shows that when production of each of these variant Tat systems was induced by the addition of arabinose, cells grew more slowly than when the wild-type Tat system was overexpressed, with the TatBL9Q substitution having a particularly detrimental effect on growth rate. This indicates that some level of toxicity is associated with overproduction of these variants. We next assessed whether the TatB variants facilitated membrane permeability using an osmotic lysis method previously used to monitor solute movement through the Sec protein transport channel (48). Here spheroplasts containing wild type or variant Tat translocases were diluted into an iso-osmotic solution of the uncharged sugar xylitol and permeation of xylitol into the cells was assessed by monitoring turbidity associated with osmotically-induced spheroplast lysis. Spheroplasts expressing a SecY variant that is known to increase permeability (48, 49) rapidly lysed following dilution into xylitol solution (Fig 7B).

However, no lysis was observed for spheroplasts producing any of the variant Tat translocases, even those harboring the TatA-oligomerizing TatB F13Y variant (Fig 7B). Western blotting confirmed that the Tat proteins were present in these membranes (Fig 7C). These results show the TatA assemblies induced by the TatB F13Y substitution do not result in a small molecule leak across the cytoplasmic membrane.
In this work a genetic approach has been taken to shed light on functions of twin arginine signal peptides during Tat transport. Two complementary screens, the first to identify substitutions in TatB permitting export of substrates with inactivating substitutions at the signal peptide arginine pair, and the second to identify rescue mutations of the TatC signal peptide binding site converged on a similar group of tatB suppressors. Four TatB substitutions were identified – three in the transmembrane domain, L9Q, L10P and F13Y, and one in the amphipathic helix (I36N) that restored Tat transport in the presence of the inactivating TatC F94Q substitution. The same three substitutions in the transmembrane helix could suppress inactivating substitutions at E103, also in the signal peptide binding site. Of these three, the F13Y substitution displayed the strongest suppressing activity, allowing export all of the twin arginine substitutions tested, and even allowing some translocation of AmiA completely devoid of a signal sequence.

A combined bioinformatics and mutagenesis approach has shown the TatB transmembrane helix to bind along transmembrane helix 5 of TatC (50), and this is consistent with in vitro disulfide crosslinking studies (25, 39). Signal peptide binding to the TatBC complex is suggested to cause movement of TatB from its resting state binding site on TatC to a site elsewhere on the protein. This is proposed to prime TatA to occupy the same binding site, which in turn triggers assembly of further TatA molecules to form the active translocase (50; Fig 8). After our biochemical experiments revealed that the suppressors did not function by restoring detectable binding of signal peptides to the TatBC complex (Fig 4, S9), we considered whether the TatB substitutions were mimicking the substrate-driven conformational changes which prime the translocase for TatA recruitment, but in the absence of substrate binding. Our analysis using BN-PAGE showed that the TatB L9Q, L10P and F13Y substitutions caused conformational alterations in the resting TatBC complex (Figure 5A,B). The complex containing the L10P substitution appeared more labile as very little full-sized TatBC complex could be detected, whereas the L9Q and F13Y substitutions yielded a subset
of TatBC complexes with apparently increased mass which may be indicative of altered subunit composition or significant conformational change. Substrate-induced conformational changes in the wild-type complex can also be monitored by appearance of a crosslink between cysteine residues at position 205 at the periplasmic end of TatC transmembrane helix 5. This residue forms part of the TatB resting-state binding site (50), so is occluded from dimerization with a neighboring TatC molecule in the resting state, but has been shown to dimerize in response to overproduction of a Tat substrate (39). Similarly, assembly of TatA-YFP oligomers (indicating assembled translocation sites) which can be monitored by fluorescence microscopy is only seen for the wild-type translocase upon overproduction of Tat substrates (34). For one of the TatB substitutions, F13Y, both TatC M205C dimerization and TatA-YFP assembly were observed not only in the absence exogenous substrates, but also in the presence of a TatC F94Q substitution which disrupts interaction with signal peptides. We therefore conclude that TatB F13Y has decreased affinity for the ‘resting’ TatC binding site, and an increased affinity for the ‘activated’ binding site, such that it is able to trigger recruitment of TatA and transport of precursors in the absence of signal peptide binding. For the other TatB variants we propose that each differs in affinity for the resting and activated binding sites, leading to slight differences in conformation for these translocases, and we assume that in these cases, weak residual binding of a signal peptide lacking its twin arginine motif is sufficient to trigger TatA recruitment, whereas in the wild-type system the higher energy of binding of the twin arginine residues are strictly required for this. Hence the signal peptide plays two distinct roles- in precursor targeting and translocase activation.

A favored model for Tat-mediated protein translocation is that protein passage across the membrane is facilitated by bilayer disruption arising from TatA oligomerization. Interestingly, it was noted that there was a reduced growth rate associated with overproduction of Tat systems containing TatB suppressors, including F13Y, suggesting apparent toxicity. However, no leak of the small uncharged sugar, xylitol, could be detected in membranes harboring Tat complexes containing TatB F13Y. This may suggest the presence of a substrate precursor is
necessary to provide the force required to disrupt the bilayer. Alternatively, it remains possible that the foci of TatA-YFP observed in cells producing TatBF13Y do not correspond to fully assembled translocases and that further recruitment of TatA(-YFP) (for example mediated by the folded mature domain of a Tat substrate (51)) is required. Indeed the export of mature AmiA in the presence of the TatB F13Y substitution might suggest that some Tat substrates have internal targeting information.

In summary, our findings support the notion that Tat signal peptides have two distinct roles. They serve to target their passenger domains to the export machinery, but also to trigger assembly of the active translocase. The isolation of substitutions in the Tat machinery that bypass these steps should prove very useful to dissect the mechanism by which folded protein translocation is achieved.
Materials and Methods

Strain construction. The *E. coli* strains used in this work are listed in SI Appendix, Table S3.

Strain JM109 was used for regular cloning and transformation of Quickchange products, and ultracompetent cells of XL10-Gold® (Agilent) were used for construction of the random mutagenesis libraries.

Strain DADE (as MC4100, ΔtatABCD, ΔtatE (42)) was used as the background strain for Tat transport activity tests and production of Tat proteins for membrane protein extraction, in vivo disulfide crosslinking and Blue-Native PAGE, with the exception of Fig 3 where strain MΔBC (MC4100 ΔtatBC; 33) was used. Strain DADE-P (as DADE, *pcnB1 zad*-981::Tn10d (Kan'); (52)) was used to co-produce TatB and TatC along with AmiA for co-purification experiments.

Strain MCDSSAC ΔtatABC (37), in which the 2-33 codon of *amiA* and 2-32 codons for *amiC* are deleted and the *tatABC* operon was replaced with an apramycin resistance cassette, was used as the background strain for AmiA signal sequence library screening and to analyse transport of AmiA mediated by AmiA or SufI signal peptide variants.

Transport of AmiA mediated by signal sequence truncations was assessed in strain MC4100 ΔamiA ΔamiC ΔtatABC, which was constructed as follows. The ΔamiA:kan' allele from the Keio collection (53) was moved into MC4100 by phage P1 transduction, after which the kanamycin resistance cassette was eliminated according to (54). Subsequently the amiC deletion was introduced and the kanamycin cassette subsequently eliminated using the same approach. Finally, the ΔtatABC::Apra allele was introduced from strain BW25113 ΔtatABC::Apra (54) by P1 transduction. Strain BW25113 ΔglpF ΔtatABC was used in osmotic lysis experiments and was constructed by P1 transduction of the ΔglpF:kan' allele from the Keio collection (53), elimination of the kanamycin resistance and P1 transduction of the ΔtatABC::Apra allele as described above.

Strain AyBCE (34), which lacks *tatA* at the native locus and has a *tatA-YFP* fusion integrated into the chromosomal *att* site, was used in fluorescence imaging. Chromosomal point
substitutions in \textit{tatB} and \textit{tatC} were introduced into this strain via plasmid pMAK-AupBC and its variants using the approach of Hamilton \textit{et al.} (55).

Strain BL21(DE3) \textit{ΔtatABC} was used to co-produce TatB and TatC along with Suflss-GFPhis for the co-purification experiments. This strain is a derivative of BL21(DE3) where the \textit{tatABC} genes have been replaced with the apramycin resistance cassette, and was constructed by recombination as described previously (56).

**Plasmid construction.** The plasmids used and constructed in this work are listed in SI Appendix, Table S4. All point mutations in plasmids, as well as insertion of the flag sequence to create p101C*BCflag, were introduced by Quickchange site-directed mutagenesis (Stratagene) using the primers listed in SI Appendix, Table S5.

Plasmids pTAT101 (39) and pTAT1d (38) were used to express \textit{tatABC} under the control of the native \textit{tatA} promoter at very low and medium copy number, respectively. pTAT101 cys less (47) was used as the backbone to introduce single cys substitutions for \textit{in vivo} disulfide crosslinking experiments. Plasmid pTATBC1d encodes TatBC and was constructed following amplification of \textit{tatBC} pTAT1d using primers STIPE-ISH and pT7.5R (SI Appendix, Table S5) was digested using \textit{Bam}H\textit{I} and \textit{Pst}I and cloned into similarly digested pUNIPROM (57).

Plasmid pBADTatABChis codes for \textit{tatABC} with a hexahistag coding sequence at the 3' end of \textit{tatC} in pBAD24 (58). It was constructed following amplification of \textit{tatABChis} from pUNITAT2 (59), digestion with \textit{Nco}I and \textit{Xba}I and cloning into similarly digested pBAD24.

pSUAmiA (36) was used to produce full-length AmiA from a vector specifying chloramphenicol resistance. pSUf\textit{lss-mAmiA} was used to produce Suflss-mAmiA and was constructed following separate amplification of DNA encoding the Sufl signal sequence including the \textit{sufI} ribosome binding site using primers SuflssFE and SuflssR, and the mature region of AmiA (mAmiA) using primers AmiA-mF and AmiA-mRX from the chromosome, and fusing the two fragments by overlap extension PCR according to reference (60). The resultant DNA fragment was then cloned into the pSU18 vector (61) using \textit{EcoR}I and \textit{Hind}III sites to generate pSUf\textit{lss-mAmiA} plasmid. Plasmids pSUf\textit{lss-noH-mAmiA} were used for production of
truncated SufISS-mAmiA lacking the signal peptide h-region. It was constructed by removal of
codons 11-21 of the SufI signal sequence via Quickchange using pSUSSufISS-mAmiA as
template with primers SufI-noHF and SufI-noHR. pSUmAmiA was used to produce signal-less
AmiA and was constructed as follows. A DNA fragment containing the ribosome binding site
of amiA and the coding sequence for mature AmiA was amplified using pSUAmiA as template
with primers AmiA-nossFE and AmiA-mRX. The DNA fragment was subsequently cloned into
pSU18 using EcoRI and HindIII sites to generate pSUAmiA. To express the mature domains
of AmiA or AmiC from pQE70, DNA covering these regions were amplified with primer pairs
mAmiA-SphI-F/AmiAnostopBamHI-R or mAmiC-SphI-F/AmiCnostopBamHI-R, respectively,
using chromosomal DNA as template. The DNA fragments were digested with SphI and
BamHI and cloned into SphI/BglII digested pQE70 vector (Qiagen, Manchester, UK). For
fractionation experiments, SufI was produced with a C-terminal histag from pQE80 (Qiagen,
Manchester, UK). It was cloned by excision of DNA covering a C-terminally his-tagged SufI
from pQE60-SufI (62) as an NheI-Xhol fragment and ligation into similarly digested pQE80.
Plasmid pMAK-AupBC was used to introduce the mutations into the AyBCE chromosome and
was constructed by amplification of 500 bp of tatA upstream DNA from the chromosome of
strain AyBCE using primers TatAup1-XbaI and TatAup2-ClaI, which was cloned into
pBluescript KS(+) using XbaI and ClaI sites to give pKS-Aup. Next a DNA fragment covering
the whole of tatBC was amplified from the chromosome of AyBCE strain with primers
TatA6B7-ClaI and TatCrev-KpnI, and cloned into pKS-Aup using ClaI and KpnI sites to
generate pKS-AupBC. Subsequently the DNA covering the tatA upstream sequence along
with tatBC was excised using XbaI and KpnI and cloned into similarly digested pMAK705 (55)
to give pMAK-AupBC.
Plasmid pFAT75ΔA-BC, which is a pQE-based plasmid expressing TatB and TatC without a
histag (19), and pSUfISS-GFPhis, which is a pCFDuet-based plasmid expressing synthetic SufI
signal sequence-fused GFP with a histag at its C-terminus under the control of T7 promoter,
were used in co-purification experiments. Plasmid pFAT75ΔA-BC-AmiAhis coproduces
untagged TatBC along with his-tagged AmiA and was constructed as follows. A DNA fragment
covering amiA was amplified from MC4100 genomic DNA using primers AmiAFATApaI-F and AmiAnostopBamHI-R, digested with ApaI and BamHI and cloned into ApaI - BglII digested pFAT75-SufIhis (47). pFAT75ΔA-BC-mAmiAhis was constructed similarly, using primer mAmiAFATApaI-F and AmiAnostopBamHI-R to amplify DNA covering the mature region of AmiA.

Mutant library construction and screening.

To screen for Tat signal sequence suppressors, substitutions of the twin arginine sequence of the AmiA signal peptide (to -RD, -RE, -RN, -RQ, -RH, -HH, -KH and –KQ) were constructed in the pSUAmiA plasmid. The resulting plasmids were individually introduced into strain MCDSSAC ΔtatABC and each resulting strain served as host to screen an existing tatB mutant library (in plasmid pTAT1d, 600,000 individual clones, 0.25% error rate, (38)).

To screen for suppressors of the TatC F94Q substitution, three separate mutagenesis libraries, each of which carried the tatC F94Q codon substitution, were constructed that contained random mutations in either tatAB, codons 1-93 of tatC (tatC1) or codons 95-258 of tatC (tatC2), respectively using a modified MEGAWHOP (megaprimer PCR of whole plasmid) method as described (63). DNA fragments of tatAB, tatC1 and tatC2 containing random mutations were generated using error-prone PCR. Error-prone PCR was carried out in 1x GoTaq buffer, 7mM MgCl2, 0.2 mM dATP, 0.2mM dGTP, 1mM dCTP, 1mM dTTP, 0.4 μM each primer, 0 to 0.1 mM MnCl2, 50 ng pTAT1dCF94Q plasmid as template and 5 U GoTaq® DNA Polymerase (Promega) in a total volume of 50 μl using a PCR program: 94 °C for 2min followed by 20 cycles of incubation at 94 °C for 30s, 50 °C for 30s, and 72 °C for 3min, and a final incubation at 72 °C for 5min. Primer pairs TatA-FB and TatB-RS were used to amplify tatAB, TatCm6 and TatC93R to amplify tatC1 and TatC95F and TatCR1d were used to amplify tatC2. The DNA fragments were then used as megaprimers to amplify the whole plasmid, which was carried out in a 50 μl mixture containing 1x Herculase II reaction buffer, 0.5 mM each dNTP, 100ng pTAT1dCF94Q plasmid as template, 500 ng DNA fragment obtained above as megaprimers and 5 U Herculase II Fusion DNA Polymerase (Agilent) using the PCR.
program: incubation at 68 °C for 5min, 95 °C for 2min, followed by 20 cycles of incubation at
95 °C for 30s, 55 °C for 30s, and 68 °C for 6min. The resultant whole plasmid PCR products
were digested with Dpn I to remove the template DNA and incubated with T4 polynucleotide
kinase and T4 DNA ligase to repair the nicks. Finally, the whole plasmids were separately
transformed into XL10-Gold® Ultracompetent Cells (Agilent) resulting in three mutagenesis
libraries. Subsequent sequencing of 10 randomly selected colonies from each library revealed
an average error rate of approximately 2 nucleotides per 1000 base pair. Screening of these
three libraries was carried out in strain DADE (ΔtatABCD, ΔtatE).

For screening experiments the libraries were transformed into the respective host strains and
subsequently plated onto solid LB medium containing appropriate antibiotics and 2% SDS for
selection. Colonies able to grow under these conditions were isolated, the mutations in the tat
gene(s) identified by sequencing and retested for growth in the presence and absence of 2%
SDS. To further verify isolated candidates, individual mutations were introduced into low copy
number plasmid pTAT101 by site-directed mutagenesis and the activity was again assessed
on 2% SDS-containing plates.

Protein methods. For co-purification of TatBC-substrate complexes, cultures of strain
BL21(DE3) ΔtatABC harboring pFAT75ΔA (or a point-substituted variant) and pSufI-GFPHis
were incubated at 37°C for 7 hours with shaking, after which they were supplemented with
0.2mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for a further 17 hours at
37°C. The cells were subsequently harvested and resuspended in 1 x PBS, the fluorescence
intensity of the suspension was recorded, after which the cells were re-pelleted, resuspended
in 2 x lysis buffer (100 mM NaH2PO4 pH 8.0, 600 mM NaCl, 40 mM imidazole, 50 mg lysozyme,
80 U benzonase, and protease inhibitor) and mixed gently at room temperature for one hour.
Cells were snap frozen at -80°C, thawed at room temperature and an equivalent amount of
2.5% digitonin was added and the sample gently mixed at room temperature for one hour. Cell
debris was pelleted by centrifugation and the supernatant was transferred to a 96-well plate
and mixed with 20 μl Ni-NTA Magnetic Agarose Beads (Qiagen) for one hour. After the beads
were washed three times with wash buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 40 mM imidazole, 0.03 % digitonin), bound proteins were eluted with 50 μl elution buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 250 mM imidazole, 0.03 % digitonin).

In vivo disulfide crosslinking was carried out in strain DADE harboring pTAT101 cys less CM205C, as described in (47). Blue-Native PAGE was undertaken according to (47, 64). Subcellular fractionation was according to (65). Preparation of membrane fractions was as described previously (39). For analysis of SufI export, E. coli strain DADE harboring wild-type or signal peptide variants of pQE80SufIhis alone or alongside wild-type or TatB variants of pTAT101 was cultured in the presence of 1mM IPTG until OD₆₀₀ of 1 was reached. Samples (equivalent to 150 μl of whole cells from an OD₆₀₀ = 1, or periplasm fractions from the equivalent of 300 μl of cells from an OD₆₀₀ = 1) were separated by SDS PAGE and analysed by Western blot with anti-6X His tag® or anti-RNA polymerase β subunit antibodies (cytoplasmic control protein). For analysis of CueO export, strain MΔBC harbouring wild-type and KK variants of pQE80-CueO alongside wild-type, tatB⁸EK or tatB⁸¹³Y variants of p101C*BCflag were cultured and fractionated as previously described (34). Immunoblotting was according to the methodology of (66), and antibodies to TatA, TatB and TatC have been described previously (47, 67). Anti-6X His tag® antibody (HRP-conjugated) was purchased from Abcam (Cambridge, UK, catalog number ab184607), anti-DnaK mouse monoclonal 8E2/2 antibody was also from Abcam (catalog number ab69617) and a mouse monoclonal anti RNA polymerase β-subunit antibody was purchased from NeoClone Biotechnology (Madison, USA; catalog number W0023). Secondary antibodies were goat anti-Rabbit IgG (HRP Conjugate, catalog number 170-6515) or Goat Anti-Mouse IgG (HRP conjugate, catalog number170-6516), both from Biorad (Hemel Hempstead, UK). Immunoreactive bands were visualized with the Clarity Western ECL Substrate Kit (BioRad) and captured either on light-sensitive film or using the GeneGNOME camera (Syngene).
**Cell permeability experiments.** Cell permeability experiments were performed according to (48). Briefly, cells were grown aerobically at 37°C with 1:100 inoculation of an overnight culture for 2 hours. Production of TatABC harboring TatB variants and of SecY(Δplug)EG was induced by addition of 0.2% arabinose at 37°C for 3 hours. Subsequently, the OD$_{600}$nm of each sample was normalized using LB, a small volume was withdrawn for Western blotting and equal volumes of each culture were then harvested and resuspended in fractionation buffer (50 mM Tris-HCl buffer, 20% sucrose, pH 7.5, 5 mM EDTA, 0.6 mg/ml lysozyme) and incubated at room temperature for 20 min to obtain spheroplasts. The spheroplast samples were then adjusted to the same OD$_{600}$ and a 19-fold excess of 0.616 M xylitol solution was added. The samples were rapidly transferred to a 96-well plate and OD$_{600}$ was measured every 30 seconds for 300 seconds.

**Fluorescence microscopy.** Cells were prepared for fluorescence microscopy and imaged as previously described (47), with the exception that a 550 nm LP emission filter was used.
Acknowledgements

This work was supported by the UK Biotechnology and Biological Sciences Research Council (through grants BB/L002531/1 and BB/N014545/1), the UK Medical Research Council (through grants G1001640, K000721 and MR/L000776/1), the Wellcome Trust (through Investigator Award 107929/Z/15/Z to BCB and a Wellcome Trust PhD studentship to SR) and the China Scholarship Council (through a studentship to QH). TP and SML are Wellcome Trust Investigators and TP is a Royal Society/Wolfson merit award holder. We thank Dr Bérengère Ize for her assistance in constructing pQE70-mAmiA and pQE70-mAmiC, Professor Ian Collinson for the gift of pBADplugSecY and Drs Hajra Basit and Mark Wallace for fluorescence microscopy access and advice.
Figure 1. Isolation of signal sequence suppressors in tatB. A. Schematic representation of a twin arginine signal peptide. The signal peptide sequences of E. coli Tat substrates SufI and AmiA are given underneath, with residues matching the Tat consensus motif in red, the consecutive arginines in red underline and the signal peptidase cleavage site in black underline. B and C. An example of screening results. Growth of MCDSSAC ∆tatABC coproducing the indicated TatB variants (with wild type tatA and tatC) from pTAT101, alongside B. the HH or C. RE-substituted signal peptide variants of AmiA, on LB agar supplemented with chloramphenicol and kanamycin, with or without the addition of 2% SDS as indicated. An 8μl aliquot of each strain/plasmid combination following aerobic growth to an OD$_{600}$ of 1.0 was spotted and incubated for 16 hr at 37°C.

Figure 2. Isolation of suppressors of the TatC F94Q inactivating substitution. A. Model of E. coli TatC (from (47)) showing the location of the F94 and E103 residues (in red) that form part of the signal peptide binding. B., C. and E. Growth of DADE (∆tatABCD, ∆tatE) coproducing wild type TatA alongside; B. and C. F94Q-substituted TatC or E. E103A-substituted TatC, and the indicated substitution in TatB from plasmid pTAT101 on LB agar or LB agar containing 2% SDS. D. Structure of E. coli TatB (from (68)) with the locations of the TatCF94Q suppressor substitutions shown in red. F. Strain MC4100 ∆amiA ∆amiC ∆tatABC coproducing the indicated TatB variants (with wild type tatA and tatC) from pTAT101, alongside either a signal peptide variant of SufI lacking the h-region fused to the AmiA mature domain, or the mature AmiA domain alone on LB agar or LB agar containing 1% SDS. For all growth tests, a single colony of each strain/plasmid combination was resuspended in 30μl of PBS and an 8μl aliquot was spotted onto LB agar supplemented with appropriate antibiotics, along with SDS as indicated and incubated for 16 hr at 37°C.
**Figure 3.** TatB suppressors support export of a Tat substrate with its native signal peptide. *E. coli* strains producing native levels of TatA, TatC and the indicated TatB variants, and overproducing his-tagged CueO with a wild-type (top panel) or KK-substituted (bottom panel) signal peptide were fractionated into whole cell (W), spheroplast (S) and periplasm (P) fractions. Equivalent amounts of each fraction were separated by SDS PAGE and analysed by Western blot with antibodies against CueO and the cytosolic marker DnaK.

**Figure 4.** The TatB suppressors do not restore signal peptide binding to the TatBC complex. A. C-terminally his-tagged GFP with the wild type (RR) or twin-arginine substituted SufI signal peptide at its N-terminus, as indicated, was purified by magnetic nickel beads from digitonin–treated cell extracts co-expressing TatC along with either wild type TatB or the E8K, F13Y or I36N substituted variants. B. C-terminally his-tagged GFP with the wild type SufI signal peptide at its N-terminus was purified by magnetic nickel beads from digitonin–treated cell extracts co-expressing TatB and TatC with the indicated amino acid substitutions. For A. and B. the elution fractions from each sample were normalized for GFP fluorescence and an equivalent amount of purified Sufiss-GFPhis was loaded onto SDS-PAGE (4-15% Mini-PROTEAN® TGX™ precast gradient gel) followed by western blot using TatB and TatC mixed antibodies.

**Figure 5.** A constitutive disulfide crosslink and aberrant Blue-native PAGE migration induced by a subset of TatB suppressors. A-B. Membranes from *E. coli* strain DADE (ΔtatABCD ΔtatE) producing the indicated TatB variants alongside wild-type TatA and TatC from plasmid pTAT1d were solubilized by addition of 2% digitonin and analysed by BN-PAGE (4-16% Bis-Tris NativePAGE gels) followed by Western blot with anti-TatA, anti-tatB or anti-TatC antibodies as indicated. 20 μg solubilized membrane was loaded in each lane. C. Membranes from strain DADE producing the indicated TatB variant alongside wild-type TatC from plasmid pTATBC1d were solubilized and analysed as in A-B. D. Whole cells of DADE harboring pTAT101 co-producing either wild type TatA, the M205C single cysteine variant of TatC and the indicated substitution in TatB, or wild type TatA, the TatC F94Q M205C variant
of TatC and the indicated substitution in TatB were subjected to oxidizing (O) or reducing (R) conditions. Where indicated the additional plasmid pQE80-CueO, (producing His-tagged CueO) was also present. Membranes were prepared from equal quantities of cells following treatment and equivalent amounts of material from each sample were resolved by non-reducing SDS-PAGE (12% acrylamide). TatC was visualized by western blotting using an anti-TatC antibody and CueO-His with an anti-His antibody.

Figure 6. The TatB F13Y substitution promotes constitutive oligomerisation of TatA in vivo. Fluorescence images of TatA-YFP in representative cells of A. strains AyBCE or AyBC_{F94Q}E (encoding the TatC F94Q substitution in chromosomal tatC) in the presence (pAmiA) or absence of plasmid-encoded wild type AmiA, as indicated. Production of AmiA was induced by addition of 1mM IPTG to mid-log cell cultures 45 min before harvesting. B. strains AyB_{F13Y}CE (encoding the TatB F13Y substitution in chromosomal tatB) and AyBC_{F94Q}E (encoding the TatB F13Y substitution in chromosomal tatB, alongside the TatC F94Q substitution in chromosomal tatC). Representative micrographs are shown for each sample; Scale bar: 1 μm.

Figure 7. No detectable leak of xylitol across the cytoplasmic membrane when cells produce Tat translocases harboring TatB suppressors. A. Overnight cultures of E. coli strain BW25113 ΔglpF ΔtatABC harboring pBAD24 encoding TatA and TatC-his along with wild type TatB or each of the L9Q, L10P, F13Y or I36N point substitutions, were subcultured at 1:100 dilution into fresh LB medium containing ampicillin, which was supplemented after 120 min with 0.2% of glucose or arabinose, as indicated. Growth of the strains was followed for a further 6.5h. Error bars represent standard deviation, n = 3 (biological replicates). B. The same strain and plasmid combinations as in part A, alongside BW25113 ΔglpF harboring pBAD22SecY(Δplug)EG. were subcultured and supplemented with 0.2% of arabinose as described in part A and grown for a further 3 h after which spheroplasts were prepared and incubated in the presence of xylitol. C. An aliquot of each sample producing plasmid-encoded
Tat proteins was analysed by SDS PAGE and western blotting to confirm expression of TatA, TatB and TatC-his.

**Figure 8.** Tat translocases containing TatB suppressor variants may more readily transition to the signal peptide-activated state. Top panel: Model for Tat transport. A signal peptide bound through its n-region to the cytoplasmic surface of TatC (step 1) transitions to a deep binding mode (step 2). The deep insertion of the signal peptide displaces TatB from its resting state binding site on TatC (grey arrow). TatB movement allows polymerisation of TatA to be nucleated (step 3). The substrate passes across the membrane facilitated by the TatA oligomer (step 4). Bottom panel: TatB variants that suppress signal sequence defects (represented as B*) may be more easily displaced from the resting state binding site. The TatB variants appear to be on a continuum with TatB F13Y pushing the Tat system into an assembled state (step 4), whereas Tat systems harboring the weaker suppressing variants are more likely to correspond to step 3.
References


A

Tat signal peptide

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Sufl

MSLSRRQFIQASGIALCAGAVPLKAGA

AmiA

MSTFKPLKTLTSRRQVLKAGLAALTLSMSQAIA

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AmiAss-HH

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AmiAss-RE
'Normal' Tat pathway:

1. Cytoplasm (A) binds TatC (C) in the periplasm (B).
2. TatC (C) forms a complex with TatB (B) and moves to the cytoplasm (A).
3. In the cytoplasm, TatC (C) is cleaved by a protease (A), releasing TatA (A).
4. TatA (A) is transported across the membrane, allowing TatC (C) to enter the cytoplasm (A).

In presence of TatB signal sequence suppressors:

1. Cytoplasm (A) binds TatC (C) in the periplasm (B).
2. TatC (C) forms a complex with TatB (B) and moves to the cytoplasm (A).
3. In the cytoplasm, TatC (C) is cleaved by a protease (A), releasing TatA (A).
4. TatA (A) is transported across the membrane, allowing TatC (C) to enter the cytoplasm (A).
A signal sequence suppressor mutant that stabilizes an assembled state of the twin arginine translocase

Qi Huang, Felicity Alcock, Holger Kneuper, Justin C. Deme³, Sarah Rollauer, Susan M. Lea, Ben C. Berks and Tracy Palmer

SUPPORTING INFORMATION
SI APPENDIX
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**Table S1.** Clones isolated from a *tatB* mutant library following screening for suppression of transport defects of inactive signal peptides. The BRE, BRN, BRQ, BRH, BHH, BKH or BKQ clone nomenclature signify substitutions isolated following screening against RE, RN or KQ variants of the AmiA signal peptide RR motif, respectively.

*identical clones
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**Table S2.** Clones isolated from a *tatAB* mutant library following screening for suppression of the transport defect arising from the TatC F94Q substitution.
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<td>Tet'(\Delta(mcrA)183\ \Delta(mcrCB-hsdSMR-mrr)173\ \text{endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacI}ZDM15 Tn10 \text{(Tet')}\text{ Amy Cam')}</td>
<td>Agilent</td>
</tr>
<tr>
<td>MC4100</td>
<td>F-(\Delta(lac)169\ \text{araD139 rpsL150 relA1 ptsF rbs flbB5301})</td>
<td>(1)</td>
</tr>
<tr>
<td>DADE</td>
<td>As MC4100, (\Delta\text{tatABC}, \Delta\text{tatE})</td>
<td>(2)</td>
</tr>
<tr>
<td>DADE-P</td>
<td>as DADE, (\text{pcnB1 zad-981::Tn10d (Kan')})</td>
<td>(3)</td>
</tr>
<tr>
<td>M\text{ABC}</td>
<td>MC4100 (\Delta\text{tatBC})</td>
<td>(4)</td>
</tr>
<tr>
<td>MCDSSAC(\Delta\text{tatABC})</td>
<td>MC4100, ami(A2-33) ami(C2-32), (\Delta\text{tatABC::Apra})</td>
<td>(5)</td>
</tr>
<tr>
<td>MC4100 (\Delta\text{amiA}) (\Delta\text{amiC}\ \Delta\text{tatABC})</td>
<td>MC4100, (\Delta\text{amiA}, \Delta\text{amiC}, \Delta\text{tatABC::Apra})</td>
<td>This work</td>
</tr>
<tr>
<td>AyBCE</td>
<td>MC4100 (\Delta\text{tatA, attB::P}<em>{\text{tatAtatA-EAK-eyfp}</em>{\text{A206K}}})</td>
<td>(4)</td>
</tr>
<tr>
<td>AyBCE ((\text{tatB}_{10Q}))</td>
<td>As AyBCE, (\text{tatB}_{10Q})</td>
<td>This work</td>
</tr>
<tr>
<td>AyBCE ((\text{tatB}_{10P}))</td>
<td>As AyBCE, (\text{tatB}_{10P})</td>
<td>This work</td>
</tr>
<tr>
<td>AyBCE ((\text{tatB}_{13Y}))</td>
<td>As AyBCE, (\text{tatB}_{13Y})</td>
<td>This work</td>
</tr>
<tr>
<td>AyBCE ((\text{tatB}_{36N}))</td>
<td>As AyBCE, (\text{tatB}_{36N})</td>
<td>This work</td>
</tr>
<tr>
<td>AyBCE ((\text{tatC}_{94Q}))</td>
<td>As AyBCE, (\text{tatC}_{94Q})</td>
<td>This work</td>
</tr>
<tr>
<td>AyBCE ((\text{tatB}<em>{9Q}) (\text{tatC}</em>{94Q}))</td>
<td>As AyBCE ((\text{tatB}<em>{9Q}, \text{tatC}</em>{94Q}))</td>
<td>This work</td>
</tr>
<tr>
<td>AyBCE ((\text{tatB}<em>{10P}) (\text{tatC}</em>{94Q}))</td>
<td>As AyBCE ((\text{tatB}<em>{10P}, \text{tatC}</em>{94Q}))</td>
<td>This work</td>
</tr>
<tr>
<td>AyBCE ((\text{tatB}<em>{13Y}) (\text{tatC}</em>{94Q}))</td>
<td>As AyBCE ((\text{tatB}<em>{13Y}, \text{tatC}</em>{94Q}))</td>
<td>This work</td>
</tr>
<tr>
<td>AyBCE ((\text{tatB}<em>{36N}) (\text{tatC}</em>{94Q}))</td>
<td>As AyBCE ((\text{tatB}<em>{36N}, \text{tatC}</em>{94Q}))</td>
<td>This work</td>
</tr>
<tr>
<td>BL21(DE3)(\Delta\text{tatABC})</td>
<td>BL21(DE3), (\Delta\text{tatABC::Apra})</td>
<td>This work</td>
</tr>
<tr>
<td>BW25113</td>
<td>(\text{lacI}^+ \text{rmB}<em>{114} \Delta\text{lacZ}</em>{W,16} \text{hsdR}<em>{514} \Delta\text{araBAD}</em>{A_{H33}} \Delta\text{rhaBA}<em>{D</em>{L78}})</td>
<td>(6)</td>
</tr>
<tr>
<td>BW25113 (\Delta\text{glpF}) (\Delta\text{tatABC})</td>
<td>BW25113, (\Delta\text{glpF}, \Delta\text{tatABC::Apra})</td>
<td>This work</td>
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</table>
Table S3. Strains used and constructed in this study.
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<tr>
<th>Plasmid</th>
<th>Relevant genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>pTAT101</td>
<td>Low copy number vector expressing TatABC under the control of tat promoter. Kan'</td>
<td>(7)</td>
</tr>
<tr>
<td>pTH19kr</td>
<td>Low copy-number cloning vector. Backbone of pTAT101.</td>
<td>(8)</td>
</tr>
<tr>
<td>pTAT101-BF6Y</td>
<td>As pTAT101, TatB F6Y exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-BE8K</td>
<td>As pTAT101, TatB E8K exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-BL9P</td>
<td>As pTAT101, TatB L9P exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-BL9Q</td>
<td>As pTAT101, TatB L9Q exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-BL10P</td>
<td>As pTAT101, TatB L10P exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-BF13Y</td>
<td>As pTAT101, TatB F13Y exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-BK30I</td>
<td>As pTAT101, TatB K30I exchange</td>
<td>This work</td>
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<tr>
<td>pTAT101-BL36N</td>
<td>As pTAT101, TatB I36N exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-CF94Q</td>
<td>As pTAT101, TatC F94Q exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-CF94A</td>
<td>As pTAT101, TatC F94A exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-CF94D</td>
<td>As pTAT101, TatC F94D exchange</td>
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</tr>
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<td>As pTAT101, TatC F94G exchange</td>
<td>This work</td>
</tr>
<tr>
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<td>As pTAT101, TatC F94K exchange</td>
<td>This work</td>
</tr>
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<td>As pTAT101, TatC F94P exchange</td>
<td>This work</td>
</tr>
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<td>As pTAT101, TatC F94R exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-CF94S</td>
<td>As pTAT101, TatC F94S exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-CF94Q-BF6Y</td>
<td>As pTAT101-CF94Q, TatB F6Y exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pTAT101-CF94Q-BE8K</td>
<td>As pTAT101-CF94Q, TatB E8K exchange</td>
<td>This work</td>
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<tr>
<td>pTAT101-CF94Q-BL9P</td>
<td>As pTAT101-CF94Q, TatB L9P exchange</td>
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<tr>
<td>pTAT101-CF94Q-BL9Q</td>
<td>As pTAT101-CF94Q, TatB L9Q exchange</td>
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<tr>
<td>pTAT101-CF94Q-BL10P</td>
<td>As pTAT101-CF94Q, TatB L10P exchange</td>
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<td>pTAT101-CF94Q-BF13Y</td>
<td>As pTAT101-CF94Q, TatB F13Y exchange</td>
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<tr>
<td>pTAT101-CF94Q-BK30I</td>
<td>As pTAT101-CF94Q, TatB K30I exchange</td>
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<td>pTAT101-CF94Q-BL36N</td>
<td>As pTAT101-CF94Q, TatB I36N exchange</td>
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<td>pTAT101-BL9Q F13Y</td>
<td>As pTAT101, TatB L9Q, F13Y exchange</td>
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<td>As pTAT101, TatB L10P, F13Y exchange</td>
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<td>pTAT101-BL9Q I36N</td>
<td>As pTAT101, TatB L9Q, I36N exchange</td>
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<td>pTAT101-BL10P I36N</td>
<td>As pTAT101, TatB L10P, I36N exchange</td>
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<td>pTAT101-CE103A</td>
<td>As pTAT101, TatC E103A exchange</td>
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<td>As pTAT101-CE103A, TatB L9Q exchange</td>
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<tr>
<td>pTAT101-CE103A-BL10P</td>
<td>As pTAT101-CE103A, TatB L10P exchange</td>
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<td>As pTAT101-CE103A, TatB I36N exchange</td>
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<tr>
<td>pTAT101-CE103K</td>
<td>As pTAT101, TatC E103K exchange</td>
<td>(7)</td>
</tr>
<tr>
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<td>As pTAT101-CE103K, TatB L9Q exchange</td>
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<tr>
<td>pTAT101-CE103K-BL10P</td>
<td>As pTAT101-CE103K, TatB L10P exchange</td>
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</tbody>
</table>
pTAT101-CE103K-BF13Y
As pTAT101-CE103A, TatB F13Y exchange
This work

pTAT101-CE103K-BI36N
As pTAT101-CE103A, TatB I36N exchange
This work

pTAT101-CP48L
As pTAT101, TatC P48L exchange
(9)

pTAT101-CP48L-BL9Q
As pTAT101-CP48L, TatB L9Q exchange
This work

pTAT101-CP48L-BL10P
As pTAT101-CP48L, TatB L10P exchange
This work

pTAT101-CP48L-BF13Y
As pTAT101-CP48L, TatB F13Y exchange
This work

pTAT101-CM59K
As pTAT101, TatC M59K exchange
(9)

pTAT101-CM59K-BL9Q
As pTAT101-CM59K, TatB L9Q exchange
This work

pTAT101-CM59K-BL10P
As pTAT101-CM59K, TatB L10P exchange
This work

pTAT101-CM59K-BF13Y
As pTAT101-CM59K, TatB F13Y exchange
This work

pTAT101-CV145E
As pTAT101, TatC V145E exchange
(9)

pTAT101-CV145E-BL9Q
As pTAT101-CV145E, TatB L9Q exchange
This work

pTAT101-CV145E-BL10P
As pTAT101-CV145E, TatB L10P exchange
This work

pTAT101-CV145E-BF13Y
As pTAT101-CV145E, TatB F13Y exchange
This work

pTAT101-CD211K
As pTAT101, TatC D211K exchange
This work

pTAT101-CD211K-BL9Q
As pTAT101-CD211K, TatB L9Q exchange
This work

pTAT101-CD211K-BL10P
As pTAT101-CD211K, TatB L10P exchange
This work

pTAT101-CD211K-BF13Y
As pTAT101-CD211K, TatB F13Y exchange
This work

pTAT101-CQ215K
As pTAT101, TatC Q215K exchange
This work

pTAT101-CQ215K-BL9Q
As pTAT101-CQ215K, TatB L9Q exchange
This work

pTAT101-CQ215K-BL10P
As pTAT101-CQ215K, TatB L10P exchange
This work

pTAT101-CQ215K-BF13Y
As pTAT101-CQ215K, TatB F13Y exchange
This work

pTAT101 cys less
As pTAT101, All 4 cys codons in tatC substituted with ala
(9)

pTAT101 cys less CM205C
As pTAT101 cys less, TatC M205C exchange
(9)

pTAT101 cys less BL9Q CM205C
As pTAT101 cys less CM205C, TatB L9Q exchange
This work

pTAT101 cys less BL10P CM205C
As pTAT101 cys less CM205C, TatB L10P exchange
This work

pTAT101 cys less BF13Y CM205C
As pTAT101 cys less CM205C, TatB F13Y exchange
This work

pTAT101 cys less BI36N CM205C
As pTAT101 cys less CM205C, TatB I36N exchange
This work

pTAT101 cys less CF94Q M205C
As pTAT101 cys less CM205C, TatC F94Q exchange
This work

pTAT101 cys less BL10P CF94Q M205C
As pTAT101 cys less CF94Q M205C, TatB L10P exchange
This work
pTAT101 cys less
BF13Y CF94Q M205C
As pTAT101 cys less CF94Q M205C, TatB F13Y
This work

pQE80-CueO
As pQE80, carrying cueO_his
(4)
pQE80-CueO\textsuperscript{R3K,R4K}
As pQE80-CueO, CueO R3K, R4K exchange
(4)
pTAT1d
Medium copy number vector expressing TatABC
under the control of tat promoter. Ampr.
(10)
pUNIPROM
pT7.5 vector carrying a tat promoter. Backbone of
pTAT1d
(11)
pTAT1d-CF94Q
As pTAT1d, TatC F94Q exchange
This work
pTAT1d-CF94A
As pTAT1d, TatC F94A exchange
This work
pTAT1d-CF94D
As pTAT1d, TatC F94D exchange
This work
pTAT1d-CF94G
As pTAT1d, TatC F94G exchange
This work
pTAT1d-CF94K
As pTAT1d, TatC F94K exchange
This work
pTAT1d-CF94P
As pTAT1d, TatC F94P exchange
This work
pTAT1d-CF94R
As pTAT1d, TatC F94R exchange
This work
pTAT1d-CF94S
As pTAT1d, TatC F94S exchange
This work
pTAT1d-CF94Q-BL9Q
As pTAT1d-CF94Q, TatB L9Q exchange
This work
pTAT1d-CF94Q-BL10P
As pTAT1d-CF94Q, TatB L10P exchange
This work
pTAT1d-CF94Q-BF13Y
As pTAT1d-CF94Q, TatB F13Y exchange
This work
pTAT1d-CF94Q-BI36N
As pTAT1d-CF94Q, TatB I36N exchange
This work
pTATBC1d
pUNIPROM carrying tatBC
This work
pSUAmiA
pSU18 carrying amiA
(12)
pSUAmiA-RD
As pSUAmiA, R14D exchange
This work
pSUAmiA-RE
As pSUAmiA, R14E exchange
This work
pSUAmiA-RH
As pSUAmiA, R14H exchange
This work
pSUAmiA-RN
As pSUAmiA, R14N exchange
This work
pSUAmiA-RQ
As pSUAmiA, R14Q exchange
This work
pSUAmiA-KH
As pSUAmiA, R13K, R14H exchange
This work
pSUAmiA-KQ
As pSUAmiA, R13K, R14Q exchange
This work
pSUAmiA-HH
As pSUAmiA, R13H, R14H exchange
This work
pSUAmiA
As pSUAmiA, R13H, R14H exchange
This work
pSUAmiA
pSU18, carrying SufI\textsuperscript{R6I}
This work
pSUAmiA-RD
As pSUAmiA, SufI R6D exchange
This work
pSUAmiA-RE
As pSUAmiA, SufI R6E exchange
This work
pSUAmiA-RH
As pSUAmiA, SufI R6H exchange
This work
pSUAmiA-RN
As pSUAmiA, SufI R6N exchange
This work
pSUAmiA-RQ
As pSUAmiA, SufI R6Q exchange
This work
pSUAmiA-KH
As pSUAmiA, SufI R5K, R6H exchange
This work
pSUAmiA-KQ
As pSUAmiA, SufI R5K, R6Q exchange
This work
pSUAmiA-KK
As pSUAmiA, SufI R5K, R6K exchange
This work
pSUAmiA-HH
As pSUAmiA, SufI R5H, R6H exchange
This work
pSUAmiA
As pSUAmiA, amiA Δ2-34
This work
pFAT75ΔA-BC
As pQEABC, but with tatA gene in frame deleted
(13)
pFAT75ΔA-BC
As pFAT75ΔA-BC, TatB E8K exchange
This work
pFAT75ΔA-BC
As pFAT75ΔA-BC, TatB F13Y exchange
This work
pFAT75ΔA-BC
As pFAT75ΔA-BC, TatB BI36N exchange
This work
pFAT75ΔA-BC
As pFAT75ΔA-BC, TatC F94Q exchange
This work

pFAT75ΔA-BC
BE8K
pFAT75ΔA-BC
BF13Y
pFAT75ΔA-BC
BI36N
pFAT75ΔA-BC
This work
pFAT75ΔA-BC CF94Q
As pFAT75ΔA-BC CF94Q, TatB L9Q exchange This work

pFAT75ΔA-BC BL9Q CF94Q
As pFAT75ΔA-BC CF94Q, TatB L10P exchange This work

pFAT75ΔA-BC BL10P CF94Q
As pFAT75ΔA-BC CF94Q, TatB F13Y exchange This work

pFAT75ΔA-BC BF13Y CF94Q
As pFAT75ΔA-BC CF94Q, TatB I36N exchange This work

pFAT75ΔA-BC-BL9Q CF94Q
As pFAT75ΔA-BC also producing C-terminally his-tagged AmiA This work

pFAT75ΔA-BC-AmiAhis CF94Q
As pFAT75ΔA-BC-AmiAhis, AmiA R14D exchange This work

pFAT75ΔA-BC-AmiARDShis CF94Q
As pFAT75ΔA-BC-AmiAhis, AmiA R14N exchange This work

pFAT75ΔA-BC-AmiARNhis CF94Q
As pFAT75ΔA-BC-AmiAhis, AmiA R13K, R14K exchange This work

pFAT75ΔA-BC-AmiAKKhis CF94Q
As pFAT75ΔA-BC-AmiAhis, AmiA R13K, R14Q exchange This work

pFAT75ΔA-BC-AmiAKQhis CF94Q
As pFAT75ΔA-BC-AmiAhis, AmiA R14D exchange This work

pFAT75ΔA-BC-mAmiAhis CF94Q
As pFAT75ΔA-BC also producing C-terminally his-tagged mature AmiA This work

pFAT75ΔA-BC-BF13YC-AmiAhis CF94Q
As pFAT75ΔA-BC, TatBF13Y exchange This work

pFAT75ΔA-BC-BF13YC-AmiARDShis CF94Q
As pFAT75ΔA-BC-BF13YC-AmiAhis, AmiA R14D exchange This work

pFAT75ΔA-BC-BF13YC-AmiARNhis CF94Q
As pFAT75ΔA-BC-BF13YC-AmiAhis, AmiA R14N exchange This work

pFAT75ΔA-BC-BF13YC-AmiAKKhis CF94Q
As pFAT75ΔA-BC-BF13YC-AmiAhis, AmiA R13K, R14K exchange This work

pFAT75ΔA-BC-BF13YC-mAmiAhis CF94Q
As pFAT75ΔA-BC-BF13YC-mAmiAhis, TatBF13Y exchange This work

pFAT75ΔA-BC-BF13YC-mAmiAKQhis CF94Q
As pFAT75ΔA-BC-BF13YC-mAmiAhis, TatBF13Y exchange This work

pQE70-mAmiA CF94Q
pQE70 producing C-terminally his-tagged mature AmiA This work

pQE70-mAmiC CF94Q
pQE70 producing C-terminally his-tagged mature AmiC This work

pSuflsss-GFPhis CF94Q
As pCDFDuet-1, carrying synthetic Sufl signal sequence-fused GFPhis This work

pSuflsSSRD-GFPhis CF94Q
As pSuflsss-GFPhis, Sufl R6D exchange This work

pSuflsSSRN-GFPhis CF94Q
As pSuflsss-GFPhis, Sufl R6N exchange This work

pSuflsSSK-K-GFPhis CF94Q
As pSuflss-GFPhis, Sufl R5K, R6K exchange This work

pQE80 sulflhis CF94Q
pQE80 carrying sulflhis This work

pQE80 RDsuflhis CF94Q
pQE80 sulflhis Sufl R6D exchange This work

pQE80 RNSuflhis CF94Q
pQE80 sulflhis Sufl R6N exchange This work

pQE80 KQsuflhis CF94Q
pQE80 sulflhis Sufl R5K, R6Q exchange This work

pMAK705 CF94Q
Cloning vector with a temperature-sensitive replicon (14)

pMAK-AupBC CF94Q
As pMAK705, carrying 500 bp upstream sequence of tatA and tatBC sequence This work

pMAK-AupBC-BL9Q CF94Q
As pMAK-AupBC, TatB L9Q exchange This work

pMAK-AupBC-BL10P CF94Q
As pMAK-AupBC, TatB L10P exchange This work

pMAK-AupBC-BF13Y CF94Q
As pMAK-AupBC, TatB F13Y exchange This work
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<thead>
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<th>Plasmid Name</th>
<th>Description</th>
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<tr>
<td>pMAK-AupBC-BI36N</td>
<td>As pMAK-AupBC, TatB I36N exchange</td>
<td>This work</td>
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<tr>
<td>pMAK-AupBC-CF94Q</td>
<td>As pMAK-AupBC, TatC F94Q exchange</td>
<td>This work</td>
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<tr>
<td>pMAK-AupBC- BL9Q CF94Q</td>
<td>As pMAK-AupBC, TatB L9Q, TatC F94Q exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pMAK-AupBC- BL10P CF94Q</td>
<td>As pMAK-AupBC, TatB L10P, TatC F94Q exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pMAK-AupBC- BF13Y CF94Q</td>
<td>As pMAK-AupBC, TatB F13Y, TatC F94Q exchange</td>
<td>This work</td>
</tr>
<tr>
<td>pBAD24</td>
<td>Arabinose-inducible protein expression vector</td>
<td>(15)</td>
</tr>
<tr>
<td>pBADTatABChis</td>
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**Table S5.** Oligonucleotides used in this study
Supplementary References


Supplementary Figure Legends

**Figure S1. Substitutions of the twin arginines in the AmiA signal peptide prevent growth in the presence of SDS.** Strain MCDSSAC ΔtatABC producing wild type tatABC from plasmid pTAT1d and either wild type (‘RR’) or signal peptide point-substituted AmiA, as indicated, from pSUAmiA. The strain and plasmid combinations were cultured overnight in LB medium supplemented with chloramphenicol and ampicillin (for plasmid selection), after which they were streaked onto LB agar containing the same antibiotics, with and without the addition of 2% SDS and incubated for 16 hr at 37°C.

**Figure S2. TatB variants are able to restore Tat transport to a range of defective twin arginine substitutions in the AmiA signal sequence.** Growth of MCDSSAC ΔtatABC coproducing the indicated TatB variants (with wild type tatA and tatC) from pTAT101, or the empty plasmid pTH19kr (indicated by ‘Δtat’) alongside signal peptide variants of AmiA, on LB agar supplemented with chloramphenicol and kanamycin, with and without the addition of 2% SDS as indicated. An 8μl aliquot of each strain/plasmid combination following aerobic growth to an OD600 of 1.0 was spotted and incubated for 16 hr at 37°C. A. Individual signal peptide substitutions of AmiA (indicated to the left of each panel) were tested against the TatB suppressors F6Y, L9P, L9Q, L10P, F13Y, K30I and I36N. B. The TatB E8K suppressor was tested for the ability to suppress the indicated AmiA signal peptide substitutions.

**Figure S3. TatB variants are able to restore Tat transport to a range of defective twin arginine substitutions in the SufI signal sequence.** Growth of MCDSSAC ΔtatABC coproducing the indicated TatB variants (with wild type tatA and tatC) from pTAT101, or the empty plasmid pTH19kr (indicated by ‘Δtat’) alongside signal peptide variants of SufI fused to the AmiA mature domain, on LB agar supplemented with chloramphenicol and kanamycin, with and without the addition of 2% SDS as indicated. An 8μl aliquot of each strain/plasmid combination following aerobic growth to an OD600 of 1.0 was spotted and incubated for 16 hr at 37°C. A. Individual signal peptide substitutions of AmiA (indicated to the left of each panel) were tested against the TatB suppressors F6Y, L9P, L9Q, L10P, F13Y, K30I and I36N. B. The
TatB E8K suppressor was tested for the ability to suppress the indicated AmiA signal peptide substitutions.

**Figure S4. A subset of amino acid substitutions at TatCF94 abolish Tat activity when produced at medium and low copy number.** A and C. Growth of DADE coproducing either wild type TatABC (Tat’), wild type TatAB alongside F94-substituted TatC or the cognate empty plasmid (Tat-) on LB agar containing 2% SDS. A single colony of each strain/plasmid combination was resuspended in 30μl of PBS and an 8μl aliquot was spotted onto LB agar supplemented with appropriate antibiotics, along with 2% SDS as indicated. Plates were incubated for 16 hr at 37°C. B and D. Detection of TatC protein present in membrane fractions of the same strain and plasmid combinations as in A. and C., respectively, by Western immunoblot with anti-TatC antiserum. A total of 5μg membranes was loaded per lane for TatC produced from pTAT1d (B) and 20μg per lane for membranes produced from strains harboring pTAT101 derivatives (D).

**Figure S5. TatB variants cannot suppress TatC inactivating substitutions outside of the signal peptide binding site.** Growth of DADE (ΔtatABCD, ΔtatE) coproducing wild type TatA alongside and the indicated substitution in TatB alongside either of TatC P48L, TatC M59K, TatC V145E, TatC D211K or TatC Q215K as indicated, from plasmid pTAT101 on LB agar or LB agar containing 2% SDS. A single colony of each strain/plasmid combination was resuspended in 30μl of PBS and an 8μl aliquot was spotted onto LB agar supplemented with appropriate antibiotics, along with 2% SDS as indicated, and incubated for 16 hr at 37°C.

**Figure S6. The suppressive effect of the TatB variants is not additive and mature AmiC is not exported in the presence of the TatB F13Y suppressor.** A. Growth of DADE coproducing either wild type TatABC (Tat’), wild type TatAB alongside F94-substituted TatC or the cognate empty plasmid (Tat-) on LB agar or LB agar containing 2% SDS. B. Growth of MCDSSAC ΔtatABC coproducing the indicated TatB variants (with wild type tatA and tatC) from pTAT101, or the empty plasmid pTH19kr (indicated by ‘Δtat’) alongside the RN or KK...
signal peptide variants of Sufl fused to the AmiA mature domain, as indicated, on LB agar with or without the addition of 2% SDS. C. Strain MC4100 ΔamiA ΔamiC ΔtatABC coproducing either wild-type TatB or TatB F13Y (with wild type tatA and tatC) from pTAT101 and the AmiA or AmiC mature domains (from pQE70-mAmiA or pQE70-mAmiC, respectively) on LB agar or LB agar containing 2% SDS. In each case a single colony of each strain/plasmid combination was resuspended in 30μl of PBS and an 8μl aliquot was spotted onto LB agar supplemented with appropriate antibiotics, along with 2% SDS where indicated. Plates were incubated for 16 hr at 37°C.

**Figure S7. The TatB suppressors support export of his-tagged Sufl with its native signal peptide.** A. and B. *E. coli* strain DADE producing wild type TatA and TatC and the indicated TatB variants alongside wild-type Sufl-his or the indicated signal-peptide variants were fractionated into whole cell (upper panels) and periplasm (lower panels) fractions, then analysed by Western blot with anti-6X His tag® or anti-RNA polymerase β subunit antibodies (cytoplasmic control protein). wc – whole cell.

**Figure S8. TatBC and Suflss-GFP-His twin-arginine variants are detectable in whole cell samples.** A. and B. Cells producing Suflss-GFP-His with the wild type (RR) or twin-arginine substituted Sufl signal peptide, as indicated, alongside TatC and either wild type TatB or the E8K, F13Y or I36N substituted variants, or C. and D. Cells producing Suflss-GFP-His with the wild type Sufl signal peptide along with either wild type TatBC, the TatC F94Q allele along with either wild type TatB or the L9Q, L10P, F13Y or I36N substituted variants, or the TatC E103K allele along with either wild type TatB or the L9Q, L10P, F13Y or I36N substituted variants, as indicated were harvested and resuspended in PBS. A. and C. The fluorescence intensity and OD600 of the samples were measured using a plate reader and the Fluorescence/OD600 plotted for each sample. B. and D. 20 μl of each cell suspension was taken, all samples were normalized to the same OD600 and then analysed by SDS-PAGE followed by western blot using a TatB-TatC mixed antibody.
**Figure S9.** TatBC complexes containing the TatB F13Y suppressor do not co-purify with signal peptide variants of AmiA. C-terminally his-tagged wild type AmiA, twin-arginine substituted AmiA or signal sequence-less AmiA, as indicated was co-produced alongside wild type TatBC or TatBF13Y/TatC and purified using nickel beads from digitonin–treated cell extracts. Aliquots of the load and elution fractions were subject to SDS-PAGE followed by Western blot using either anti-His, anti-TatB and TatC antibodies.

**Figure S10.** TatB variants are extracted from the membrane with digitonin. Membrane suspensions (containing equivalent amounts of total protein) from strain DADE co-producing either wild type TatABC or wild type TatA and TatC alongside the indicated amino acid variant of TatB were solubilized by addition of 2% digitonin and incubation on ice for 30 min. Samples total membranes and digitonin solubilized material (each containing 10[$\mu$]g protein) were analysed by SDS-PAGE followed by western blotting with anti-TatA, anti-TatB or anti-TatC antibodies as indicated.

**Figure S11.** Constitutive oligomerisation of TatA is not promoted by the TatB L9Q, L10P or I36N substitutions. Fluorescence images of TatA-YFP in representative cells of A. strains AyBCE or AyBC$_{F94Q}$E (encoding chromosomal TatC F94Q) in the presence (pAmiA) or absence of plasmid-encoded wild type AmiA, as indicated (reproduced from Fig 5A). B. strains AyB$_{L9Q}$CE (encoding chromosomal TatB L9Q), AyB$_{L10P}$CE (encoding chromosomal TatB L10P) and AyB$_{I36N}$CE (encoding chromosomal TatB I36N) or the same strains additionally harboring the chromosomally-encoded TatC F94Q substitution. Scale bar: 1 [$\mu$m]. Note that the pictures in panel A are identical to those in Fig 5A and were included here to provide a direct comparison with panel B.
Fig S1
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**Fig S2**
Fig S3
Fig S4
Fig S5
Fig S6
Fig S7
Fig S8
Fig S9
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Fig S10
Fig S11