The *Escherichia coli* twin-arginine translocase: conserved residues of TatA and TatB family components involved in protein transport

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Abstract The *Escherichia coli* Tat system serves to export folded proteins harbouring an N-terminal twin-arginine signal peptide across the cytoplasmic membrane. In this report we have studied the functions of conserved residues within the structurally related TatA and TatB proteins. Our results demonstrate that there are two regions within each protein of high sequence conservation that are critical for efficient Tat translocase function. The first region is the interdomain hinge between the transmembrane and the amphipathic α-helices of TatA and TatB proteins. The second region is within the amphipathic helices of TatA and TatB. In particular an invariant phenylalanine residue within TatA proteins is essential for activity, whereas a string of glutamic acid residues on the same face of the amphipathic helix of TatB is important for function.

Key words: Protein transport; Tat pathway; Twin-arginine signal peptide; Trimethylamine *N*-oxide reductase; Site-directed mutagenesis

1. Introduction

The translocation of proteins across the bacterial cytoplasmic membrane normally proceeds by one of two pathways. The classical Sec pathway is the major route of protein translocation. Substrate proteins are threaded through the Sec translocon in an unfolded state, driven by the energy of ATP hydrolysis [1,2]. However, in many bacteria, translocation may proceed by a second route. This alternative mode of protein translocation is by the Tat pathway. Proteins are directed for export by the Tat machinery by N-terminal signal peptides that harbour an (S/T)-R-R-x-F-L-K twin-arginine motif, where the consecutive arginine residues are normally invariant [3]. The Tat system is structurally and mechanistically related to the ΔpH-dependent import pathway of plant thylakoid membranes [4]. The key mechanistic feature of the Tat system is that it exports pre-folded proteins, often containing redox-active cofactors, without rendering the membrane permeable to protons and other ions [5,6].

In *Escherichia coli*, four genes encode membrane-bound components of the Tat machinery [7]. Three of these, *tatABC*, are organised in an operon with a fourth gene, *tatD*, that is not involved in Tat-dependent protein translocation [8]. The *tatE* gene is located elsewhere on the chromosome. The protein products of the *tatA* and *tatE* genes are more than 60% identical, and previous work has shown them to share the same function [7]. However, *tatE* is transcribed and translated at a 100 fold lower rate than *tatA* and is considered to be a cryptic gene duplication [9]. TatA has a single transmembrane domain at its N-terminus, which is immediately followed by an amphipathic α-helix that is also capable of associating with the membrane [10,11]. It has a C-terminal domain of some 40 amino acids that is dispensable for protein translocation [12]. Both genetic and biochemical experiments indicate that TatA is the major protein component of the Tat machinery since it is expressed at about a 20 fold higher level than TatB or TatC [9,13]. TatA when expressed alone has been shown to form a large homo-oligomeric complex, and predominantly cross-links to itself even in the presence of other Tat components [10,11].

The *tatB* gene encodes a protein that has a similar structural arrangement to, and approximately 20% identity with, TatA. The functions of TatA and TatB in Tat-dependent protein export are, however, distinct [14]. TatB is absolutely required for the export of all naturally occurring *E. coli* substrate proteins tested, but there is some indication that the artificial substrate TorA-ColV may still be translocated in the absence of TatB [15]. Cross-linking experiments indicate that TatB exists minimally as a homodimer in cell membranes [10]. TatB has been shown to form a complex with TatA, and also to comprise part of a separate complex with TatC [13,16,17]. The TatC protein is the most highly conserved of all of the Tat components. It is highly hydrophobic, with either 4 or 6 transmembrane spans [18,19]. It has been implicated, along with TatB, as the site of recognition of the twin-arginine signal peptide [20,17].

TatA proteins are smaller than TatB proteins having a shorter amphipathic helical region and a shorter carboxy-terminal tail. It is likely that the sequence constraints on the TatB family are stricter than those on the TatA family since *Helicobacter pylori* TatA is able to complement an *E. coli tatA/tatE* mutant, whereas the *H. pylori* TatB cannot rescue the *E. coli tatB* deletion strain [14]. However, sequence analysis alone is not always sufficient to assign a protein to the TatA or TatB family and some bacteria, for example *Rickettsia prowazekii* and *Staphylococcus aureus* only possess a single protein of the TatA/B family [5,21]. In this work we

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have sought to further explore the properties of TatA and TatB proteins. Using site-directed mutagenesis we have examined the roles of conserved residues in TatA and TatB. Our results suggest that residues at the junction of the hydrophobic and amphipathic helices are important for the function of both proteins. Moreover, an absolutely conserved phenylalanine residue in the amphipathic region of TatA and a string of glutamic acid residues in the amphipathic helix of TatB are essential for effective translocation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli strains MC4100 (F$^-$Δ*lacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301*; [22]), MC4100-P (As MC4100 *pcn*B1 *zad*-981::Tn*10*d (Km^r); [23]), JARV16-P (As MC4100 Δ*tatA*Δ*tatE pcn*B1 *zad*-981::Tn*10*d (Km^r); [14]) and BØD-P (As MC4100 Δ*tatB pcn*B1 *zad*-981::Tn*10*d (Km^r); [14]) were employed in this study. During all genetic manipulations, *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium [24]. Concentrations of antibiotics were as described previously [7]. The growth phenotypes of mutants with trimethylamine *N*-oxide (TMAO) as sole respiratory electron acceptor were determined on M9 minimal medium agar plates supplemented with 0.5% glycerol and 0.4% TMAO and incubated in a gas jar under a hydrogen/carbon dioxide atmosphere [23]. The SDS-resistance phenotype of mutants was tested on LB agar plates containing 2% SDS [23]. For TMAO reductase assay, cells were cultured in modified Cohen and Rickenberg medium [25], supplemented with addition of 0.4% glucose and 0.4% TMAO.

2.2. Plasmid construction

Plasmid pFAT415 carries the *tatA* gene and more than 500 bp of upstream DNA in pBluescript [14]. Plasmid pFAT416 carries more than 500 bp of DNA sequence upstream of *tatA* (as in pFAT415) but also the Δ*tatA* allele and the intact *tatB* gene in pBluescript [14]. Site-specific mutations in pFAT415 and pFAT416 were constructed by polymerase chain reaction methods or using the QuikChange™ system (Stratagene) (primer sequences available on request). The E8A allele of *tatB* was introduced directly into pFAT75 [13] using the QuikChange™ method. For cross-complementation analysis, a number of *tatA* and *tatB* mutant alleles were subcloned from the pBluescript plasmid into the polylinker of the compatible plasmid pSU18 [26] after digestion with *Xba*I and *Kpn*I.

All clones obtained from PCR amplified DNA were sequenced to ensure that no mutations (other than site-specific mutations) had been introduced.

2.3. Protein methods

SDS–PAGE and immunoblotting using anti-TatA and anti-TatB antibodies were carried out as described previously [10] and immunoreactive bands were visualised with the ECL detection system (Amersham Pharmacia Biotech). Membrane preparation and cross-linking was as described previously [10].

Subcellular fractions for TMAO reductase activity measurements were prepared from small (30 ml) cultures using the cold osmotic shock protocol [27], or using the lysozyme-EDTA method [7]. TMAO:benzyl viologen oxidoreductase activity was measured as described previously [28]. Protein concentrations were estimated according to the method of Lowry et al. [29].

3. Results

3.1. Site-directed mutagenesis of TatA

The *E. coli tatA* gene encodes a protein of 89 amino acids, with a molecular weight of 9.6 kDa. It is generally not straightforward to use multiple sequence alignments to identify those sequence features that distinguish a TatA protein from a TatB protein [5,14,21]. This is because it is often unclear whether a given prokaryotic TatA/B homologue is functionally a TatA or a TatB protein. Such assignments can,

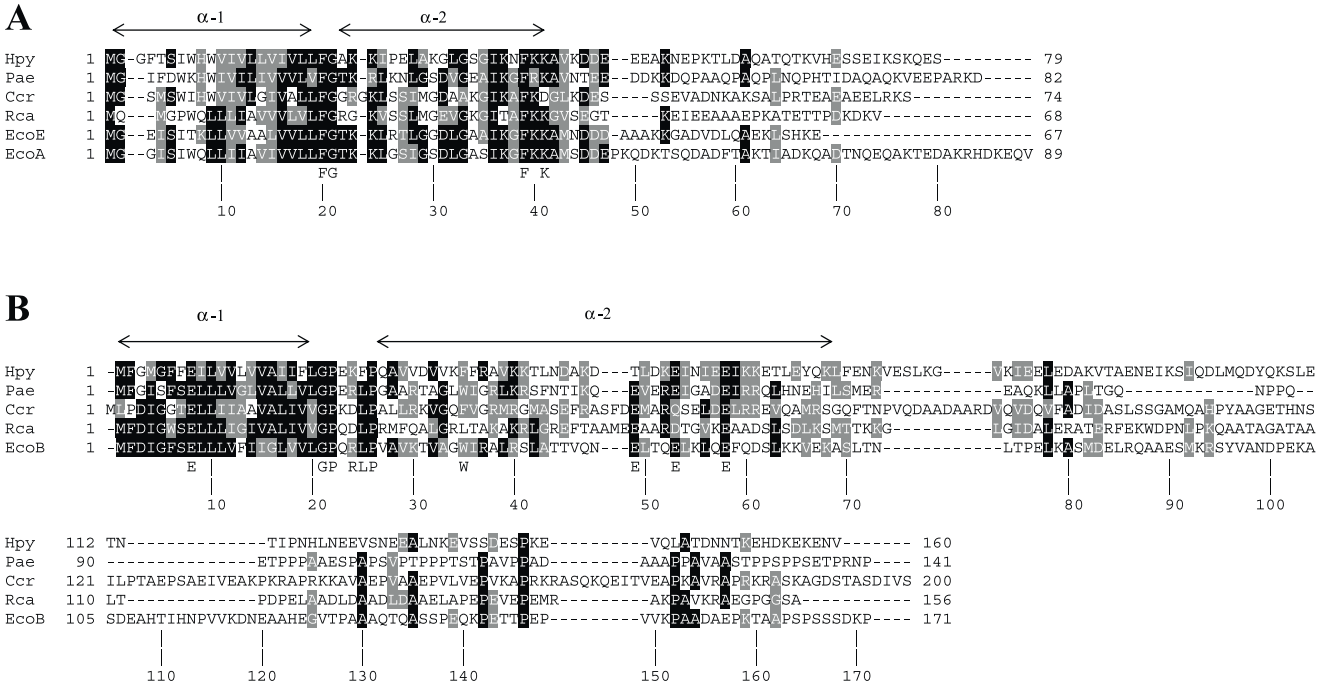


Fig. 1. Sequence alignment of TatA and TatB family proteins. A: Alignment of TatA proteins. B: Alignment of TatB proteins. Amino acid sequences of a representative cross-section of TatA and TatB family proteins were aligned by CLUSTAL W1.8 and shaded with BOXSHADE. The positions of the transmembrane (α-1) and amphipathic (α-2) helical regions are depicted above the amino acid sequence. The numbers shown beneath each alignment refer to the *E. coli* TatA or TatB protein respectively, and amino acid residues that were mutated in this study are also shown below each alignment. Abbreviations are Eco – *Escherichia coli*, Pae – *Pseudomonas aeruginosa*, Hpy – *Helicobacter pylori*, Ccr – *Caulobacter crescentus*, Rca – *Rhodobacter capsulatus*. The A and E after Eco in Fig. 1A denote TatA and TatE sequences, respectively. Residues that are found in 60% or more of the sequences are blocked in black. Conservative substitutions are blocked in grey.

however, be made with confidence if the analysis is restricted to the Proteobacteria (which includes *E. coli*) and a comparison of TatA (and TatE) sequences from a number of proteobacteria is shown in Fig. 1A. The absolute amino acid conservation amongst the TatA proteins is low, and the sequence homology extends only to the end of the amphipathic helix. The lack of amino acid conservation beyond residue 40 is consistent with our previous observation that the C-terminal 40 residues of TatA are not essential for Tat-dependent protein export [12]. Of the highly conserved amino acids, the phenylalanine residue at position 20 (*E. coli* nomenclature) is invariant, as is the glycine at position 21. Gly21 falls at the boundary between the hydrophobic and amphipathic helices. It is the only residue that is absolutely conserved in both the TatA and TatB protein families, and has been proposed to act as a hinge between the two helices [5]. Other strictly conserved residues in TatA are a phenylalanine at residue 39, and the charged residue (normally lysine) at position 41.

We assessed the importance of these residues by substituting each in turn with alanine. These substitutions were expressed on a multicopy plasmid in a strain deleted for *tatA* and *tatE*, and which also carries the *pcnB* allele that we have shown previously to significantly reduce plasmid copy number [23]. As seen in Fig. 2A, most of the substitutions had a severe effect on the export of the Tat substrate TMAO reductase (TorA) to the periplasm. Thus the F20A, G21A and F39A substitutions gave very low levels of periplasmic TorA activity. As a second test, we assessed the ability of each of these mutant plasmids, when expressed in the *tatA*/*E* deletion strain, to support growth on minimal medium containing TMAO as sole electron acceptor. No growth was observed with the F39A allele indicating that this mutation completely blocks TorA export. However, the other alleles permitted TMAO-dependent growth indicating that, although TorA translocation was severely affected by these mutations (Fig. 2A) it was not abolished. We have shown recently that strains with an inactive Tat system are highly sensitive to killing by the detergent SDS, due to the mislocalisation of two Tat-dependent amidase proteins [30,31]. As a third test, we assessed the ability of the *tatA*/*E* deletion strain carrying the mutant alleles to grow in the presence of 2% SDS. All of the mutations supported growth on SDS with the exception of the F39A substitution. We conclude on the basis of alanine substitution that only Phe39 of the highly conserved residues in TatA is essential for Tat-dependent export.

3.2. The TatA mutant proteins assemble into the membrane

In order to examine whether each of the mutant TatA proteins was synthesised and assembled into the membrane, we prepared membrane fractions from the *tatA*/*E* mutant strain carrying each of the *tatA* alleles. We assessed the levels of TatA protein by immunoblotting. As shown in Fig. 3A, each of the TatA mutant proteins was present in the membrane fraction at levels similar to that of the wild type TatA, with the exception of the K41A substitution. There was some K41A-substituted TatA present in the membrane, but the level was much lower than that of the other TatA proteins. This result indicates that substitution of the lysine residue at position 41 of TatA results in destabilisation of the protein.

We have previously used cross-linking analysis to demonstrate that the TatA protein is found in homo-oligomeric complexes [10]. We therefore decided to use a cross-linking approach to assess whether the stable TatA variants were affected in self-self interactions. As shown in Fig. 3B and consistent with our earlier studies [10] the reagent disuccinimidyl suberate (DSS) cross-links homodimers and homotrimers of wild type TatA in the membrane environment. Cells expressing only the G21A and F39A TatA variants showed identical patterns of cross-linking. These observations suggest that these mutations do not affect the ability of TatA to assemble into homo-oligomeric complex and would be consistent with protein engineering studies which indicate that the transmembrane helix is required for oligomerisation of TatA [11].

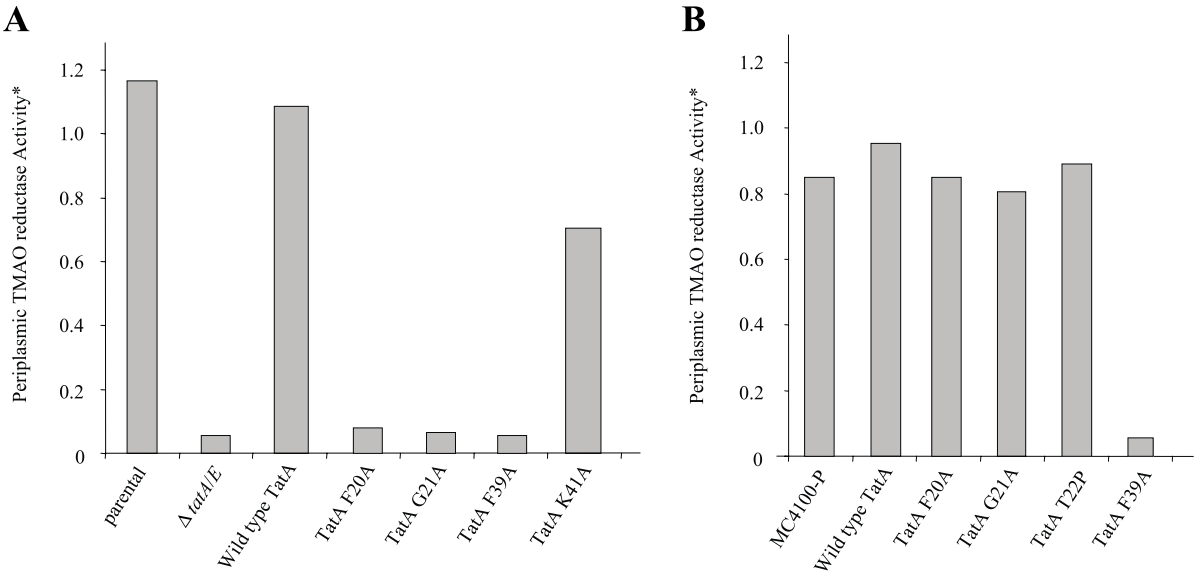
3.3. The TatA F39A mutation is dominant

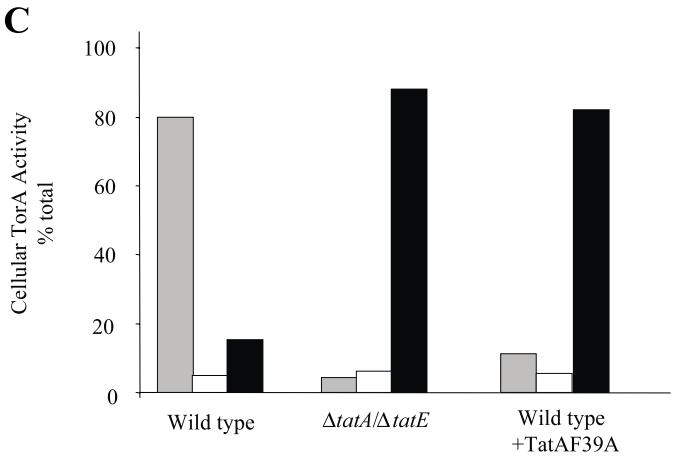
We expressed the mutants that severely affect TatA activity in a strain carrying the wild type *tat* genes to assess whether any of the mutations had a dominant negative phenotype. As shown in Fig. 2B, none of the mutations displayed dominance with the exception of the F39A-substituted TatA, which almost abolished the periplasmic TorA activity of the wild type strain. To confirm that the TorA in the wild type strain expressing this mutant form of TatA was mislocalised rather than unstable, we fractionated to obtain in addition to the periplasm, the membrane and cytoplasmic fractions. Almost all of the TMAO reductase activity was clearly mislocalised to the cytoplasm in the wild type strain expressing the TatA F39A allele (Fig. 2C). We therefore conclude that the TatA F39A mutant protein is capable of interacting with and affecting the activity of wild type Tat complexes.

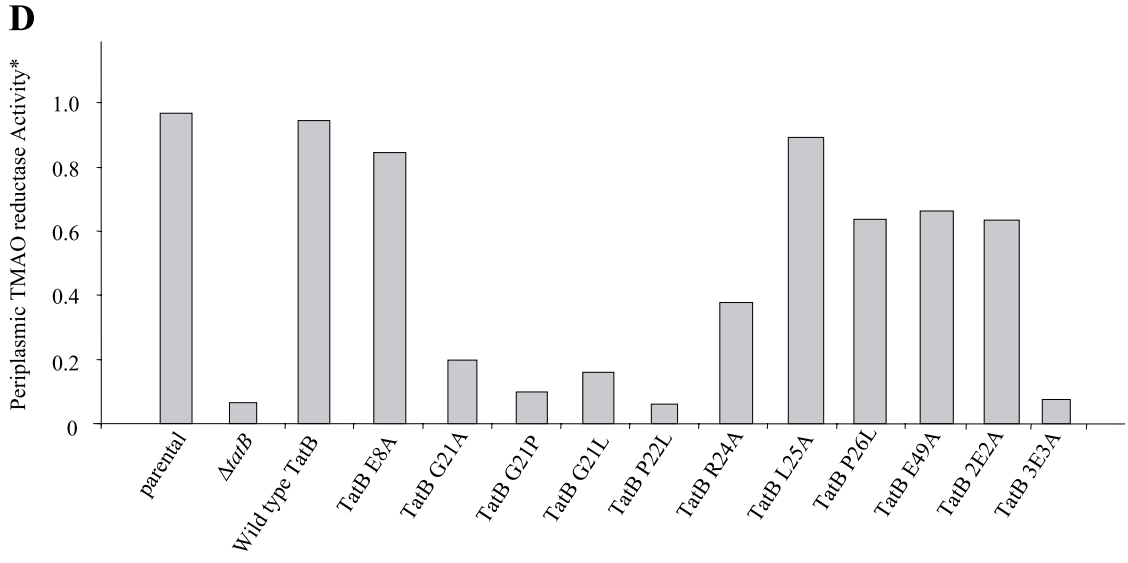
3.4. Site-directed mutagenesis of TatB

The *E. coli tatB* gene encodes a protein of 171 amino acids with a molecular weight of 18.4 kDa. Truncation analysis has indicated that the transmembrane and amphipathic α-helical regions are essential for TatB activity but that the last 70 amino acids, covering most of the C-terminal domain, are dispensable for function [12]. Comparison of a number of Proteobacterial TatB sequences, shown in Fig. 1B, reveals that the region of homology does not extend much beyond residue 60 (*E. coli* numbering). There is an absolutely conserved glutamic acid residue in the transmembrane domain (E8) that has previously been proposed to have a role in proton translocation [14]. Gly21, Pro22, Leu25 and Pro26 are found throughout the TatB family. There is also a conserved basic residue at position 24 of TatB and a string of conserved glutamates in the amphipathic region (E49, E53 and E58 in *E. coli* TatB). In order to test whether any of these residues were essential for TatB function we constructed a number of site-directed mutations. The effects of these mutations on the level of periplasmic TorA activity are shown in Fig. 2D. Surprisingly, substitution of Glu8 to alanine did not have a significant effect on the periplasmic TorA level indicating that, although it is strictly conserved, the presence of an acidic residue at this position is not necessary for TatB function. Substitution of 'hinge' Gly21 for Ala reduced the periplasmic TMAO reductase activity but the effect of this mutation was less severe than the equivalent substitution in TatA. Substitution of the TatB Gly21 with leucine or proline gave a similar phenotype to the alanine substitution. In a previous study a *pro22leu* mutation in the chromosomal copy of *tatB* was identified as being responsible for the Tat$^-$ phenotype of *E. coli* strain D-43 [32]. We analysed the same Pro22Leu substitution

in our experimental system. We found that while this allele has a profound effect on periplasmic TorA level (Fig. 2D) Tat-dependent protein translocation was not totally abolished since we observed some growth of the strain both on minimal TMAO plates and in the presence of SDS (not shown). Of the other conserved residues, substitution of Arg24 (for Ala), Leu25 (for Ala) or Pro26 (for Leu) had little effect. A single replacement of Glu49 for Ala did not significantly affect peri-







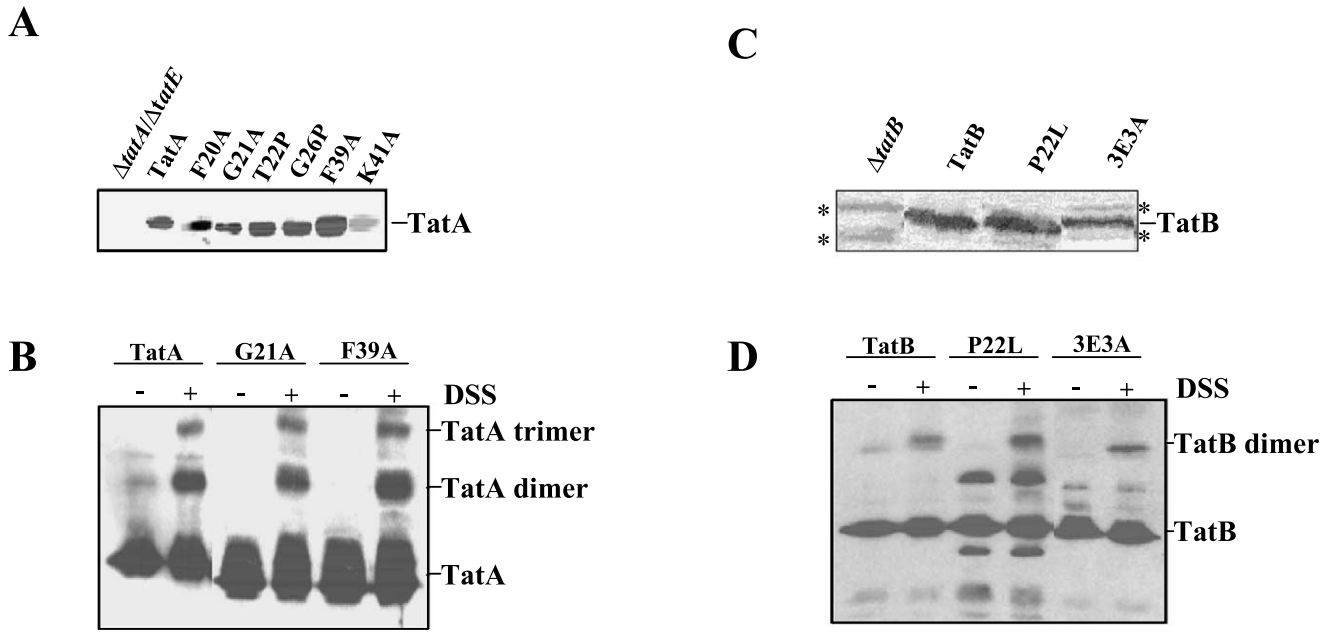


Fig. 3. The TatA and TatB mutant proteins assemble into the membrane. A: Western blot analysis of the Δ*tatA*/Δ*tatE* strain (lane Δ*tatA*/Δ*tatE*) alone or the same strain expressing wild type (lane TatA) or point mutated TatA proteins. B: Detection of TatA homo-oligomers by DSS cross-linking. Membranes were prepared from the Δ*tatA*/Δ*tatE* strain expressing wild type (lanes marked TatA) or point mutated (lanes G21A and F39A) TatA. The membranes were either left untreated (lanes 1, 3 and 5), or incubated with 2 mM DSS (lanes 2, 4 and 6), as described previously [10]. Samples were analysed by immunoblotting with anti-TatA antibodies. C: Western blot analysis of the Δ*tatB* strain (lane Δ*tatB*) alone or the same strain expressing wild type (lane TatB) or point mutated TatB proteins. The asterisks indicate non-specific bands that cross-react with the antibody. D: Detection of TatB homo-dimers by DSS cross-linking. Membranes were prepared from the Δ*tatB* strain expressing wild type (lanes marked TatB) or point mutated (lanes P22L and 3E3A) TatB. The membranes were either left untreated (lanes 1, 3 and 5), or incubated with 2 mM DSS (lanes 2, 4 and 6), as described previously [10]. Samples were analysed by immunoblotting with anti-TatB antibodies. The lanes marked 3E3A denote the TatB E49A, E53A, E58A allele.

plasmic TorA activity, nor did a double substitution (E53A and E58A; denoted as 2E2A in Fig. 2D). However, co-substitution of all three conserved acidic residues in the TatB amphipathic helix (3E3A in Fig. 2D) almost abolished periplasmic TorA activity. All of the TatB mutant plasmids were able to support growth of the Δ*tatB* strain on minimal medium containing TMAO, and on SDS plates, indicating that, although some mutations severely affected Tat transport, none was essential for Tat translocation. None of the TatB mutations exhibited dominance when expressed in a wild type strain (not shown).

3.5. The TatB mutant proteins assemble into the membrane

We prepared membrane fractions from the Δ*tatB* mutant strain expressing the TatB P22L and the triple E49A/E53A/E58A variants that are severely affected in Tat transport activity. As shown in Fig. 3C, both of the mutant proteins were present in the membrane fraction at levels similar to wild type. We have previously shown that a homo-dimer of the wild type TatB protein can be cross-linked in the membrane environment. As shown in Fig. 3D both the TatB variants retained the ability to be cross-linked as a dimer by DSS. Therefore the P22L and E49A/E53A/E58A mutations do not affect the ability of the TatB protein either to assemble into the membrane or to form dimers in the membrane environment.

3.6. Differences between TatA and TatB family proteins

Major sequence differences between TatA and TatB proteins in Proteobacteria are associated with the predicted hinge region. TatA proteins invariably have a strictly conserved Phe residue immediately preceding Gly21 whilst TatB proteins do not have the conserved Phe but instead normally have a Leu at this position. In addition TatB proteins also have the invariant proline at residue 22 (Fig. 1B). In other organisms the correlation between these amino acids and whether a protein is TatA or TatB is less clear. For example, all three of the *Bacillus subtilis* TatA/B proteins have an FGP motif, as does the chloroplast protein Hcf106 which is thought to be func-

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Fig. 2. Periplasmic TMAO reductase activities from strains expressing mutated TatA and TatB proteins. A: Periplasmic TorA activities from either MC4100 (parental strain), JARV16-P (Δ*tatA*/Δ*tatE*, *pcnB1*) or JARV16-P carrying plasmid-expressed wild type or point mutated TatA proteins. B: Periplasmic TorA activities from MC4100-P (as MC4100, *pcnB1*) carrying plasmid-expressed wild type or point mutated TatA proteins. *TMAO reductase activity in panels A, B and D is expressed as μmol benzyl viologen oxidised/min/mg protein. C: Percentage of TorA activity found in the periplasmic (grey bars), membrane (white bars) or cytoplasmic (black bars) fractions of either MC4100-P (designated 'wild type'), JARV16 (designated Δ*tatA*/Δ*tatE*) or MC4100 pTatAF39A (designated wild type+TatAF39A). Activities are expressed as a percentage of the total for each strain, which were 322, 147 and 301 μmol/min/g cells respectively. D: Periplasmic TorA activities from either MC4100 (parental strain), BØD-P (Δ*tatB*, *pcnB1*) or BØD-P carrying plasmid-expressed wild type or point mutated TatB proteins. The columns marked 2E2A and 3E3A denote the TatB E53A, E58A and TatB E49A, E53A, E58A alleles, respectively.

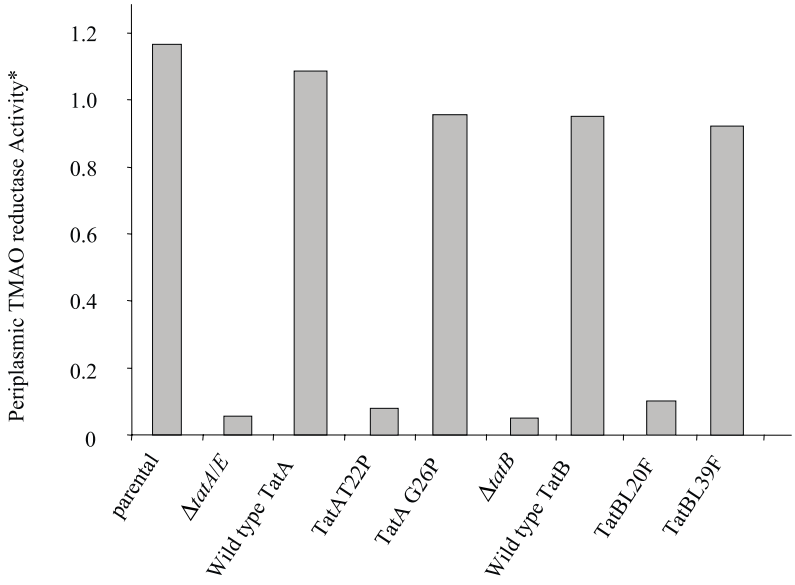

Fig. 4. Interconversion of amino acid residues between TatA and TatB family proteins. Periplasmic TorA activities from MC4100 (parental strain), from JARV16-P (Δ*tatA*/Δ*tatE*, *pcnB1*), from JARV16-P carrying plasmid-encoded wild type TatA, TatA T22P or TatA G26P mutations, from BØD-P (Δ*tatB*, *pcnB1*) or from BØD-P carrying plasmid-expressed wild type TatB, TatB L20F or TatB L39F mutations. *TMAO reductase activity is expressed as μmol benzyl viologen oxidised/min/mg protein.

tionally a TatB protein. In order to test whether these residues serve to functionally define the two families, we made a number of substitutions designed to interchange these conserved residues between TatA and TatB. Pro22 is always found in TatB proteins, but the corresponding amino acid in TatA is commonly Thr or Ala. We therefore substituted a proline for Thr22 of TatA. As shown in Fig. 4, this almost completely inactivated Tat transport showing that a proline residue cannot be functionally tolerated in this position of the TatA protein. We changed the Leu at residue 20 of TatB to Phe thus giving the same FGP motif within TatB that is found in the TatA Thr22Pro variant. As shown in Fig. 4, this mutation almost inactivated the function of TatB. These observations suggest that the amino acids adjacent to Gly21 are critical for the function of both TatA and TatB and that the specific side chain requirements are different for the two proteins.

In addition to the proline directly following Gly21 TatB has a second absolutely conserved proline at residue 26. Substitution of Gly26 of *E. coli* TatA for Pro was comfortably tolerated by TatA (Fig. 4). Likewise, introduction of the invariant Phe39 of TatA at the same position of TatB (L39A) did not significantly affect Tat-dependent transport (Fig. 4).

4. Discussion

In this study we have sought to investigate the role of conserved amino acid residues in the function of TatA and TatB proteins. To this end we have constructed a number of site-directed mutations in the most highly conserved residues of each of these proteins and assessed their effects with respect to the transport of TMAO reductase as well as the ability of the strain to grow in the presence of SDS which is a measure of the export of two Tat-dependent cell wall amidases AmiA and AmiC [31]. Most of the highly conserved residues in TatA fall within two regions of the protein, these being the proposed hinge region between the hydrophobic and amphipathic α-helices (F20 and G21), and towards the carboxy terminus of the amphipathic helix (F39 and K41). Mutations within the hinge region of *tatA* had a marked effect on Tat translocase activity. In particular increasing the size of the side chain of invariant Gly21 by one methyl group (Gly21Ala variant) was sufficient to reduce periplasmic TorA activity to less than 10% of wild type levels. Residues within the amphipathic helix of TatA were also important for TatA function. This is consistent with our previous results in which carboxy-terminal truncations of TatA that extend into the amphipathic region led to loss of TatA function [12]. Replacement of the absolutely conserved phenylalanine at residue 39 by alanine completely inactivated Tat function with respect to TorA translocation and growth on SDS. Moreover, expression of this mutation in a wild type background was sufficient to almost fully disrupt Tat activity. These results indicate that the F39A allele of TatA is able to assemble into Tat complexes, and presumably to interact with wild type copies of TatA.

The TatB protein has a similar overall organisation to TatA, and again conserved residues cluster within the inter-domain hinge and the amphipathic helical regions. However, the glycine kink at the hinge region may be reinforced in TatB proteins by two invariant prolines, one immediately following the invariant glycine residue and the second four residues later. A mutation of TatB at proline 22 had previously been reported [32]. In this report we confirm that this first proline, but not the second, is almost essential for TatB function since substitution by leucine renders TatB almost transport-incompetent. Likewise, the presence of glycine at residue 21 is necessary to support efficient translocation. Interestingly, introduction of a proline residue at an equivalent position (Thr22) of TatA almost abolished transport activity, showing that the sequence in this region of TatA and TatB proteins is a defining feature of the two protein families.

An intriguing observation is the presence of three conserved glutamic acid residues in the amphipathic helix of TatB, arranged such that they would fall exactly on the same face of a regular α-helix. These residues are clearly important for func-

tion. Although we could substitute one or two of these residues by alanine, a triple substituted TatB protein was all but inactive. This is of particular interest since it has been proposed that a complex of TatBC forms the initial site of signal peptide binding by the Tat machinery [20], and therefore this highly negative patch is a prime candidate for twin-arginine recognition during the binding event.

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