Genetic Evidence for a TatC Dimer at the Core of the *Escherichia coli* Twin Arginine (Tat) Protein Translocase

Barbara Maldonado\(^a\)  Grant Buchanan\(^a\)  Matthias Müller\(^c\)  Ben C. Berks\(^b\)
Tracy Palmer\(^a\)

\(^a\)Division of Molecular Microbiology, College of Life Sciences, University of Dundee, Dundee, and \(^b\)Department of Biochemistry, University of Oxford, Oxford, UK; \(^c\)Institute of Biochemistry and Molecular Biology, ZBMZ, Freiburg, Germany

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**Abstract**
The twin arginine protein transport (Tat) system transports folded proteins across the cytoplasmic membranes of prokaryotes and the thylakoid membranes of plant chloroplasts. In *Escherichia coli*, the TatB and TatC components form a multivalent receptor complex that binds Tat substrates. Here, we have used a genetic fusion approach to construct covalent TatC oligomers in order to probe the organisation of TatC. A fused dimer of TatC supported Tat transport activity and was fully stable in vivo. Inactivating point mutations in one or other of the TatC units in the fused TatC dimer did not inactivate TatC function, indicating that only one TatC protomer in the TatC fused dimer needs to be active. Larger covalent fusions of TatC also supported Tat transport activity but were degraded in vivo to release smaller TatC forms. Taken together, these results strongly suggest that TatC forms a functional dimer, and support the idea that there is an even number of TatC protomers in the TatBC complex.

Protein transport across the bacterial cytoplasmic membrane and the thylakoid membrane of plant chloroplasts is achieved by one of two general pathways. The Sec (general secretory) pathway exports proteins in an unfolded conformation, powered by SecA-mediated ATP hydrolysis and the protonmotive force \((\Delta p)\) [Driessen and Nouwen, 2008]. By contrast, the twin arginine protein transport (Tat) pathway transports folded proteins across the membrane, driven solely by \(\Delta p\) [Cline and Theg, 2007; Muller, 2005; Palmer et al., 2010]. Proteins are targeted to the Sec and Tat pathways by cleavable N-terminal signal peptides that share similar features; however, Tat signal peptides harbour a conserved twin arginine motif which is essential for recognition by the Tat machinery [Berks, 1996; Stanley et al., 2000].

In Gram-negative bacteria such as *Escherichia coli*, the Tat machinery comprises three membrane-bound components, TatA, TatB and TatC, orthologues of which are also found in plant thylakoids [Bosch et al., 1998; Mori et al., 2001; Sargent et al., 1998, 1999; Settles et al., 1997; Weiner et al., 1998]. The TatA protein probably forms the protein-conducting element of the Tat system. It forms large homo-oligomeric ring-like structures that surround central cavities of sufficient dimensions to accom-
modate folded proteins [Gohlke et al., 2005]. In vivo, large complexes of TatA appear to assemble from tetrameric units in a process which is dependent on the presence of the TatB and TatC components [Dabney-Smith et al., 2006; Leake et al., 2008].

The TatB protein shares structural homology with TatA but has a distinct and essential function in protein export [Sargent et al., 1999]. Together with TatC, it is a component of the TatBC complex where the two constituent polypeptides are present in 1:1 stoichiometry [Bolhuis et al., 2001]. The TatBC complex acts as a receptor for Tat substrates, interacting with them primarily through the twin arginine signal peptide, but also making contacts to the mature domain [Alami et al., 2003; de Leeuw et al., 2002; Mori and Cline, 2002; Richter and Bruser, 2005; Tarry et al., 2009; Maurer et al., 2010].

The TatBC complex contains multiple copies of TatB and TatC. The precise stoichiometry of the complex is unknown, but probably contains between 6 and 8 copies of each protein [Bolhuis et al., 2001; Oates et al., 2003; Richter and Bruser, 2005; Tarry et al., 2009]. Disulphide mapping studies have been used to infer that the TatB component is arranged as a bundle on the interior of the complex, whereas TatC is localised at the periphery [Lee et al., 2006; Punginelli et al., 2007]. Both TatB and TatC can be cross-linked to signal peptides; however, cross-linking of TatC is restricted to the twin arginine motif of the signal peptide [Alami et al., 2003; Gerard and Cline, 2006]. A role for TatC in the recognition of the twin arginine motif is supported genetically by the isolation of mutant strains that switch the specificity of transport from signal peptides containing twin arginines to twin lysines [Strauch and Georgiou, 2007].

Recently, it has been shown that the TatBC complex is multivalent. Isolation of the E. coli TatBC complex from cells overproducing the SufI substrate protein followed by analysis by negative stain electron microscopy revealed a proportion of TatBC complexes with two adjacent-bound SufI molecules [Tarry et al., 2009]. Studies of the orthologous complex (Hcf106:cpTatC) from thylakoids show that up to four substrate molecules bound to a single receptor complex can be transported together [Ma and Cline, 2010]. In this system, receptor-bound substrates can be cross-linked to each other through exposed cysteine residues [Ma and Cline, 2010], again suggesting that substrates bind at adjacent sites within the receptor complex.

In this study, we have constructed TatC fusion proteins to address questions relating to the arrangement and stoichiometry of TatC proteins in the TatBC complex. Our studies strongly suggest that the functional unit of TatC is a dimer. Moreover, by introducing inactivating point mutations into the individual TatC units of the dimer, we show that only one functional TatC within the dimeric unit is sufficient for Tat transport activity.

**A Fused TatC Dimer Is Functional**

The precise number of TatB and TatC subunits within the TatBC complex is uncertain. Size analysis of the isolated complex by gel filtration and blue native PAGE estimates between 6 and 8 copies of each protein [Bolhuis et al., 2001; de Leeuw et al., 2002; Oates et al., 2003; Richter and Bruser, 2005; Tarry et al., 2009], with a heptameric arrangement suggested from analysis of substrate-bound TatBC complexes by electron microscopy [Tarry et al., 2009]. Determining the precise stoichiometry of protein complexes is not always straightforward. One way this has been approached is to construct genetic fusions between partner proteins. This methodology was used to infer a 1:1 stoichiometry of E. coli TatB and TatC [Bolhuis et al., 2001] and was also used to probe the organisation of the c subunit in the F$_0$ portion of the E. coli H$_{\text{+}}$-transporting ATPase [Jones and Fillingame, 1998].

We therefore designed a strategy to construct genetic fusions of TatC, utilizing the tatABC overexpressing vector, pUNITAT2 [McDevitt et al., 2005], where the TatC protein is supplied with a C-terminal hexahistidine tag. An approach was used where any number of tatC genes could be fused together and is based on the premise that the restriction enzymes Bsp120I and Eco52I generate compatible ends which when ligated together results in destruction of both recognition sequences, thus allowing continuous addition of tatC units to the 3’ end. Full details of this strategy are provided in figure 1a.

Initially, we tested this approach by constructing a covalent TatC dimer. To assess whether the fused TatC dimer was competent to support Tat transport activity, it was coproduced with TatA and TatB in a strain lacking chromosomal tat genes, and the activity of trimethylamine-N-oxide (TMAO) reductase, a Tat-exported enzyme, was determined in the periplasmic fraction. It is clear from figure 1b that the fusion protein was able to support Tat transport activity. As shown in figure 2, the fused TatC dimer could also support transport of a second Tat substrate, periplasmic nitrate reductase (Nap). Thus, an E. coli tat mutant strain producing Nap as the only nitrate reductase showed similar growth kinetics on nitrate-containing medium when either the monomeric...
**Fig. 1.** A covalent dimer of TatC is functional. **a** Schematic representation of the construct expressing fused dimeric tatC. Initially, a starter unit of tatC was cloned into pLitmus28 (New England Biolabs) by amplification with oligonucleotides TatCAflII (GCGC GAATTC CTTAAGCATGTCTGTAGAAGATACT) and TatCadnaus1 (GCGC AGATCT GGGCCCTTCTTCAGTTTTTT) and digestion with EcoRI and BglII, to give pLitmus-TatC-starter. An additive unit of tatC was also cloned into pLitmus28 following amplification of tatC using oligonucleotides TatCadnaus1 and TatCadnaus2 (GCGC GAATTC CGGCCGTCTGTAAGATACCAACC) and digestion with EcoRI and BglII, to give pLitmus-TatC-buildblock. The tatC gene from pLitmus-TatC-starter was excised by digestion with AffII and BglII and cloned into similarly digested pUNITAT2 [McDevitt et al., 2005] to give pUNITAT2-TatC-starter. This construct was digested with Bsp120I and BglII, and the tatC gene from pLitmus-TatC-buildblock was cloned into this as an Eco52I-BglII fragment to give pUNITAT-TatCx2. The sequence of the fusion junction between the tandem tatC genes is shown. The sequence at the 3' end of the second tatC unit is also shown (with the Bsp120I and BglII restriction sites shown in double and single underline, respectively). Additional tatC units can be added sequentially by digestion of the vector with Bsp120I and cloning of tatC units from pLitmus-TatC-buildblock as Eco52I-BglII fragments. Note that the final tatC unit in each fusion is fused to a hexa histidine tag-coding sequence. **b** A fused dimer of TatC has Tat transport activity. Periplasmic TMAO reductase activities (assayed according to the method of Silvestro et al. [1988] from strains cultured anaerobically overnight in LB medium supplemented with 0.5% glycerol and 0.4% TMAO) were measured from the periplasmic fraction of the parental strain, MC4100-P (tatε [Buchanan et al., 2002]; designated WT) or the tatABCDE deletion strain DADE-P [Lee et al., 2006] carrying either empty pQE60 vector (Δ), or a plasmid producing TatA, TatB along with monomeric (pUNITAT2; designated TatC) or fused dimeric TatC (from pUNITAT-TatCx2; designated TatCx2). **c** The fused TatC dimer is stable. Western blot analysis (approximately 2 × 10⁷ cells loaded per lane) of whole cell samples from DADE-P producing TatA, TatB along with either monomeric or fused dimeric TatC grown under identical conditions to **b**. Samples were separated by SDS PAGE (10% acrylamide) and blotted with anti-TatC antiserum [Alami et al., 2002].
or fused dimeric TatC variant was present, and the levels of nitrite produced (the product of nitrate reduction) were also indistinguishable between the strain harbouring monomeric and fused dimeric TatC (fig. 2). These results were also backed up by the observations (data not shown) that the TatC dimer could support aerobic growth on LB medium containing 2% SDS (inactivation of the *E. coli* Tat machinery results in a sensitivity to growth in the presence of SDS due to an inability to export the cell wall amidases AmiA and AmiC [Ize et al., 2003]).

Since non-native proteins can be targets for proteolysis, we next assessed the stability of the TatC fusion protein in whole cell samples. As shown in figure 1c, a single cross-reacting band is visible in the sample containing the fused TatC dimer migrating close to the expected position for a dimeric TatC species [Punginelli et al., 2007]. Thus, we can conclude that a dimeric form of TatC is functional and stable. These findings support previous genetic evidence for a functional multimer of TatC, where certain inactive alleles of tatC could cross-complement each other [Buchanan et al., 2002], and is consistent with biochemical evidence that some cysteine-substituted TatC proteins can form crosslinked dimers [Punginelli et al., 2007]. The simplest conclusion supported by these findings is that the TatBC complex contains an even number of TatC (and therefore presumably also TatB) subunits.

**Only One TatC Unit in the Fused TatC Dimer Needs to Be Active to Support Tat Transport Activity**

The results from the previous section strongly suggest that a TatC dimer acts as a functional unit during Tat transport. To probe whether both constituent monomers within the TatC dimer are required for function, we introduced the inactivating F94A point mutation into either the first or second TatC unit in the fused dimer, or into both halves of the dimer, using standard PCR methods [Buchanan et al., 2002]. The precise function of TatC F94 is not known, although evidence points to a role in the recognition of twin arginine signal peptides by TatC. For example, it has been shown that TatBC complexes containing the monomeric TatC F94A variant are unable to cross-link to substrate precursors containing a photoactivatable amino acid located close to the twin arginine motif in the signal peptide [Holzapfel et al., 2007], and serine substitution at this position facilitates the recognition of variant signal peptides containing a normally inactive twin lysine motif [Strauch and Georgiou, 2007].

Previous studies have indicated that the F94A substitution is one of the few point mutations in TatC that completely inactivates the protein [Barrett et al., 2005; Buchanan et al., 2002; Holzapfel et al., 2007] and in agreement with this a fused TatC dimer where both units harboured the F94A amino acid substitution was unable
to support any detectable Tat transport activity. Thus, negligible TMAO reductase activity could be detected in the periplasmic fraction of a strain producing this TatC variant (fig. 3a), and the fusion protein was unable to support growth in the presence of SDS or with TMAO as sole electron acceptor (which is a consequence of the inability to export either TMAO or dimethylsulphoxide (DMSO) reductases; growth data not shown).

However, when the fused dimer of TatC carried only one inactivating F94A mutation, the Tat system appeared to show wild type activity, since the periplasmic TMAO reductase activity from strains producing either of these two variant proteins was indistinguishable from that seen for the fused dimer of wild-type TatC (fig. 3a). This was true regardless of whether the functional TatC was the first or second unit of the fused dimer. As shown in figure 3b, each of the dimeric TatC species was completely stable in vivo, ruling out the possibility that activity arose from TatC proteins that had been degraded to free monomers. These results therefore confirm that only one of the TatC units in the core dimer needs to be functional to support an active Tat system.

Recent biochemical and structural analysis of the E. coli TatBC complex indicates that it harbours at least two substrate-binding sites that from electron microscopy analysis of TatBC-SufI complexes are always located adjacently [Tarry et al., 2009]. The orthologous complex from plant thylakoids has also been shown to bind multiple precursors, presumably at several adjacent sites, which are collectively transported [Ma and Cline, 2010]. However, the findings here might suggest that adjacent functional receptor sites are not an obligate requirement for operation of the E. coli Tat pathway since inactivating one of the units in the fused TatC dimer did not appear to affect operation of the Tat pathway. An alternative explanation that we cannot rule out is that the dimeric units of TatC are arranged head-to-head such that active TatC units from two neighbouring dimers are positioned next to each other.
to each other (although such an organisation would only be feasible for an octameric rather than hexameric stoichiometry).

**The Inactive TatC Dimer Containing Two F94A Amino Acid Substitutions Does Not Show Genetic Dominance**

It has previously been shown that the inactive F94A TatC variant does not interfere with the function of wild-type TatC, i.e. the mutant allele does not show genetic dominance [Buchanan et al., 2002]. The results presented above may provide an explanation for this observation, since it is clear that only one of the TatC proteins needs to be functional for any pair of TatCs in the TatBC complex. We therefore sought to test whether a TatC in which both TatC units in the fused dimer were inactivated by the F94A substitution could disrupt the activity of native TatC. To this end, the plasmid-encoded inactive TatC variant was produced in a strain that was wild type for all chromosomal *tat* genes and the activity of TMAO reductase was determined from the periplasmic fraction of the cell. However, despite the fact that this variant completely inactivated Tat transport when it was the only form of TatC, as shown in figure 4, the inactive fused dimer of TatC did not greatly reduce the export of TMAO reductase, and therefore we conclude that it does not apparently disrupt the function of native TatC within the TatBC complex.

Taken together, these observations suggest that the dimers of TatC function as discrete units and that it is not necessary for all of the dimers within a TatBC receptor complex to be active. This is in accord with the observations of Tarry et al. [2009] who observed only one or two SufI molecules bound to each TatBC complex. In the experiments described here, it is likely that the plasmid-produced inactive TatC dimer is in considerable excess (around 40-fold over native, chromosomal, levels of TatC from this plasmid expression system [Punginelli, 2005]), and therefore these observations suggest that only very few TatBC units in any one receptor need to be functional to support wild type levels of Tat transport activity.

**Larger Fused Oligomers of TatC Support Tat Transport Activity but Are Subject to in vivo Degradation**

Since the genetic fusion approach described above provided convincing evidence that a covalent TatC dimer was functional, we sought to use the same technology to construct larger oligomeric fusions of TatC. Using the strategy outlined in figure 1a, we extended the number of repeating TatC units sequentially, up to a tandem fused hexameric TatC. We then tested the ability of the fused TatC oligomers to support Tat-dependent protein transport, again by measuring the activity of TMAO reductase from periplasmic fractions. As shown in figure 5a, it is apparent that each of the TatC fused oligomers had Tat transport activity. Furthermore, each of the TatC fusions tested could support anaerobic growth on minimal media containing TMAO as sole electron acceptor and on LB medium containing 2% SDS (data not shown).

To assess the stability of the fused oligomers, we carried out Western blotting on the same whole cell samples that we subsequently fractionated for the TMAO reductase assays. As shown in figure 5b, whilst the fused dimer of TatC was completely stable, all of the larger fusion proteins showed degradation to smaller forms.

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Fig. 4. A fused TatC dimer carrying inactivating point mutations in each TatC unit does not show dominance. TMAO reductase activities were measured from the periplasmic fraction of the *tat* strain MC4100-P carrying either empty pQE60 vector (WT) or a plasmid producing TatA, TatB along with fused dimers of TatC, where both TatC units harboured the inactivating point mutation (F94A/F94A), or from the *tatABCDE* deletion strain DADE-P carrying empty pQE60 vector (Δ) as indicated. 100% activity is that determined for MC4100-P harbouring the empty vector and the error bars represent the standard error of the mean (n = 3 or 4).
Thus, whilst Tat transport activity was noted for strains producing these fusions, we cannot rule out that the activity arose due to the release of smaller degradation products rather than being associated with the full-length fusion protein.

Whilst the instability of the TatC fusion proteins may be due to the fact that they are overproduced, it occurred to us that the degradation of the TatC fusions might also be related to protein transport by the Tat machinery. To assess this, we deleted the tatA gene from each of these TatC fusion-encoding clones (by subcloning of the ΔtatA allele from pFAT75A [McDevitt et al., 2005] as an EcoRI-EcoRV fragment) and examined the stability of the TatC proteins encoded on these constructs in strain DADE, which is deleted for all chromosomal tat genes. However, in this case we also saw significant degradation of the TatC fusions (data not shown). Thus, it appears that TatC fusions which are larger than a fused dimer are inherently unstable and that their degradation is not related to the activity of the Tat machinery.

In conclusion, the results presented here support the idea that E. coli TatC is a functional dimer, and would therefore indicate that the TatBC complex contains even numbers of subunits. The instability of larger fused repeats of TatC does not allow conclusions to be drawn about higher-order arrangement of the TatC. However, it is possible that additional functional arrangements such as trimers, tetramers or hexamers of TatC might also exist, at least transiently, during Tat transport. Determining the precise stoichiometry of the TatBC complex and indeed whether this might change during protein transport will require further experimentation.

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