Twin Arginine Translocation (Tat)-dependent Export in the Apparent Absence of TatABC or TatA Complexes Using Modified Escherichia coli TatA Subunits That Substitute for TatB*

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The twin arginine translocation pathway exports folded proteins across the thylakoid membrane of many bacteria. In Escherichia coli and other Gram-negative bacteria, TatA, TatB, and TatC are all essential for efficient translocation, and current models suggest that separate TatABC and TatA complexes coalesce at the point of translocation. However, other microbes appear only to possess tatA and tatC genes. In Escherichia coli, virtually no translocation is observed when only TatA and TatC are present, but several mutations at the extreme N terminus of TatA were shown to support translocation. Here we show that these apparently bifunctional mutant TatA variants can function as typical TatA components because translocation is observed when they are co-expressed with TatBC, and they assemble into large, heterogeneous complexes that resemble wild type TatA complexes. However, cells expressing TatC plus the mutant TatA variants do not contain complexes that resemble the expected 370-kDa TatABC complex, clearly indicating that the mutant TatA forms cannot assemble efficiently, or stably, into this complex. The simultaneous expression of wild type TatA furthermore blocks translocation activity, suggesting that the mutant TatA forms preferentially bind to other TatA molecules rather than TatC. Surprisingly, we observe translocation in the absence of detectable free TatA, when translational fusions of the mutant TatAs with TatC are expressed. Transport can thus proceed in the simultaneous absence of TatABC and TatA complexes at detectable levels, and we conclude that the active translocon may be formed from dynamic twin arginine translocation complexes, one or more of which may await characterization.

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‡ The abbreviations used are: Tat, twin arginine translocation; BN, blue-native; TMAO, trimethylamine N-oxide; TorA, TMAO reductase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; IPTG, isopropyl β-D-thiogalactopyranoside; F, forward; R, reverse.

The twin arginine translocation (Tat)2 system is used to transport proteins across the thylakoid membrane of chloroplasts or the cytoplasmic membrane of many bacteria. In contrast to the general secretory (Sec) system where proteins are threaded through the membrane in an unfolded manner, Tat substrates are translocated in a fully folded state, in a process that is believed to require a transmembrane proton gradient (reviewed in (1, 2)). Substrates of the Tat system vary greatly in size and composition, and the transport of oligomeric complexes (3), redox cofactor-containing proteins (4–6) and proteins that do not bind cofactors has been reported. These are targeted to the membrane-bound Tat apparatus via cleavable N-terminal signal peptides which contain a consensus Tat recognition motif S/T-RxFLK (7, 8).

In Escherichia coli and several other Gram-negative bacteria the Tat apparatus consists minimally of three subunits: TatA, TatB and TatC (1, 9, 10). In E. coli, TatA and TatB exhibit 25% sequence homology, but they carry out distinct functions and cannot substitute for each other (11). However, it is often unclear from sequence analysis alone whether a given protein is a TatA or TatB homolog (12). In such cases only functional characteristics can distinguish them as they have similar predicted structures, including a single transmembrane spanning α-helix with the N terminus located in the periplasm, followed by an amphipathic α-helix and a mainly unstructured C terminus (1, 2). The third gene in the Tat operon, tatC encodes for a six-span protein that has both the N and C termini located in the cytoplasm (13, 14).

The mechanism of translocation is still poorly understood, but purification and cross-linking studies have allowed the early stages to be hypothesized. Two complexes have been purified from the membranes of cells overexpressing the three essential Tat proteins. In E. coli a TatABC-containing complex was purified and shown to consist of TatB and TatC in equal amounts, with variable levels of TatA associated (15, 16). The mass of this complex was estimated as 370 kDa using blue native polyacrylamide gel electrophoresis, a technique which lessens the influence of detergent on size estimation (17). Although some TatA was found complexed with TatB and TatC, the large majority is found in separate homo-oligomeric complexes (17). More recently, the TatA complex has been studied in some detail using single-particle electron microscopy (18), and a possible role in formation of the translocation pore was suggested.

The TatABC complex is thought to be involved in the initial substrate-binding step, as cross-linking studies reveal that the
signal peptide associates with TatB and TatC and this can occur with de-energized membranes where further translocation is prevented (19, 20). Cross-links between the signal peptide and TatA are not visualized until a proton motive force is applied across the membrane, suggesting that TatA complexes are recruited following signal peptide recognition to form an active translocon. Recent studies show the importance of specific residues of TatA for homo-oligomer formation (21, 22) and have led to the hypothesis that different amounts of TatA are recruited in relation to the pore size required for specific substrates to be translocated.

TatC has been shown to be required for the recognition of the twin arginine motif of the signal peptide and TatA is involved in active translocon formation, but the precise role of TatB in E. coli has not been clearly identified. Most Gram-positive organisms contain only tatC genes (12, 23, 24) suggesting that TatA may be bifunctional in these bacteria, fulfilling both TatA and TatB roles. In support of this idea, Blaudeck et al. (25) recently showed that translocation of a chimeric Tat substrate can occur in the presence of only TatA and TatC after selecting for TatA mutants that compensate, at least to a certain degree, for the absence of TatB. The mutations were located at the extreme N terminus of TatA.

So far, the modified TatA molecules have been analyzed using a chimeric Tat substrate, and the export capabilities of these TatA/TatC mutant translocases have not been directly investigated at the protein level. Furthermore, it is not clear whether typical Tat complexes are formed. In the present study, we have characterized the Tat complexes in cells expressing these mutant TatA variants. We show that translocation can proceed in the absence of any of the previously identified Tat complexes.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions—**E. coli strain MC4100 was the parental strain; ΔABCDE has been described before (26) and arabinose-resistant derivatives were used as described (27). The Tat subunits were expressed using either the arabinose-inducible pBAD24 vector (15) or the IPTG-inducible pHSG575 vector (25, 28). E. coli were grown at 37 °C in LB broth (LB) as described (15) supplemented with glycerol (0.5% (v/v)), trimethylamine N-oxide (TMAO; 0.4% (w/v)), and sodium molybdate (1 µM). This medium is referred to as LB-GT. Medium supplements were used at the following concentrations: ampicillin, 100 µg/ml; arabinose, 50 µM; chloramphenicol, 25 µg/ml; IPTG, 1 mM unless otherwise stated.

A vector encoding TatA and TatC proteins was generated as follows. The tatA gene was amplified with the primers TatA Ncol F and TatA PstI R and the tatCs gene was amplified with the primers TatC PstI F and TatC XbaI R using a template that already contains a Strep II tag (29) at the 3’ end. These and other primers are shown in Table 1. The resulting fragments were digested with Ncol and PstI, and PstI and XbaI, respectively, and cloned in one step into pBAD24, generating pBAD-A-CFs.

A vector encoding a fusion of TatA and TatC was generated as follows. The tatA gene was amplified with the primers TatA Ncol F and TatA 10Asn PstI R and the tatCs gene was amplified with the primers TatC Xa PstI F and TatC XbaI R using a template that already contains a Strep II tag (29) at the 3’ end. These and other primers are shown in Table 1. The resulting fragments were digested with Ncol and PstI, and PstI and XbaI, respectively, and cloned in one step into pBAD24, generating pBAD-A-Cs.

Site-directed mutagenesis was used to generate constructs encoding the tat operon within pBAD-ABCs, pBAD-A+C-Cs, and pBAD-A-CFs with point mutations in the tatA gene using the QuikChange™ mutagenesis system (Stratagene) according to the manufacturer’s instructions. The mutations are denoted M1/M2 for the TatA substitutions (25), and only the forward primers are shown (Table 1).

A vector encoding wild type or modified TatA with the fusion or separate copies of wild type or modified TatA and TatC were generated as follows. Wild type or modified tatA was amplified with the primers TatA NheI F and TatA NcoI R and the mutants were sequenced, and ΔABCDE cells were transformed.

**SDS-PAGE and Immunoblotting—**Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane. Membranes were probed with antiserum raised against TatA, TatB, or the Strep II tag (IBA, Goettingen, Germany). TatA and TatB were detected using horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody, and ECL™ reagents (Amersham Biosciences). TatC was detected (via the Strep II tag) using horseradish peroxidase-conjugated anti-mouse IgG secondary antibody and ECL reagents.

**TolR Activity Assay—**Cells containing pBAD24-derived constructs were grown in LB-GT with 50 µM arabinose for 3.5 h. Cells containing pHSG575 derived constructs were grown in

**TABLE 1**

**PCR primers used in this study**

Nucleotides identical to genomic DNA are capitalized, and restriction sites are underlined. Nucleotides in boldface specify a linker consisting of 10 Asn residues; nucleotides in italics specify a factor Xa cleavage site; IEGR.

| Primer name | Sequence 5’ → 3’ |
|-------------|------------------|
| TatA Ncol F | gctacagccATACGGGTTGGTATACGTC |
| TatA PstI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC PstI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA Xa PstI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| M1 (G2SG3D) | G3GAAGTTACCATAGGAGTTTCAAACTG |
| M2 (G3D) | G3GAAGTTACCATAGGAGTTTCAAACTG |
| TatA NheI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA 10Asn PstI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA NcoI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA Ncol R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA NheI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA 10Asn PstI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA NcoI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA Ncol R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA NheI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA 10Asn PstI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA NcoI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA Ncol R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA NheI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA 10Asn PstI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA NcoI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA Ncol R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA NheI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA 10Asn PstI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA NcoI F | gctacagccggctagaATACCGCTCCTTTATCG |
| TatC XbaI R | gctacagccggctagaATACCGCTCCTTTATCG |
| TatA Ncol R | gctacagccggctagaATACCGCTCCTTTATCG |

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**Results**

An E. coli TatAC Minimal Translocase Exports an Authentic E. coli Tat Substrate—Previously, it has been shown that a combination of TatA and TatC can mediate translocation of a chimeric, cofactor-less substrate, TorA-MalE, in the absence of TatB if the extreme N terminus of TatA is modified (25). These mutant TatA subunits contain the sequences MSDIS (mutant M1) or MGDIS (mutant M2) in place of the MGGIS of the wild type.

To analyze native E. coli Tat substrates, we tested whether these TatA + TatC constructs can translocate a large cofactor-containing protein, TMAO reductase (TorA). TorA folds and binds its molybdopterin cofactor in the cytoplasm, before being exported to the periplasm (33). The TatA + TatC constructs were expressed in *tat* null mutant ΔABCDE cells, which were then fractionated, and active TorA was visualized by a native gel activity assay (30, 34). Fig. 1A shows that in the absence of Tat components (ΔABCDE) all of the TorA activity is localized in the cytoplasm as expected (lane C). When wild type TatABC is expressed (from plasmid pHSG-ABC) most of the TorA activity is located in the periplasm, confirming that the Tat system is active. The cytoplasmic form sometimes runs as a slower migrating band in these gels, for unknown reasons. As expected, when wild type TatA and TatC are expressed in the absence of TatB (Fig. 1A, lane A + C), no translocation of TorA to the periplasm is detected. In contrast, the modified versions of TatA (M1 and M2), are able to export TorA. These data demonstrate that only the TatC and modified TatA subunits are required to translocate a large native *E. coli* protein, and that the translocase formed must have the flexibility to accept differently sized substrates (MalE and TorA are 42 and 86 kDa, respectively). The assay is not quantitative, but it appears that the mutant TatA-TatC combinations support less efficient export than TatABC because a more prominent cytoplasmic TorA signal is usually observed.

Although these constructs are able to translocate Tat substrates to the periplasm, they are derived from a low copy plasmid, pHSG575, which caused reduced levels of Tat components in the membrane (data not shown). Therefore, we cloned TatA and TatC into the arabinose-inducible vector pBAD24, which has been shown to express the tatABC operon at higher levels (>10-fold) compared with wild type TatABC levels (15). A Strep II tag was fused to the C terminus of TatC during cloning to enable detection (the tag does not inhibit the function of the Tat translocase (15)). In the figures, this tagged TatC is abbreviated to Cs; for example expression of TatA mutant M1 with Strep II-tagged TatC equates to (M1 + Cs), and expression of wild type TatA plus tagged TatC is denoted by (A + Cs).

First, we analyzed the TatABC levels in cells expressing each of the different TatA mutants together with TatC, using antibodies specific to TatA, TatB, or the Strep II tag. The results are shown in Fig. 1B. In control samples, no Tat components are visualized in the membranes from ΔABCDE cells, although they are clearly detected when wild type TatABC is expressed from pBAD-ABCs. Expression of the three TatA +
TatA-TatC Containing Tat Complexes

The Presence of TatB Does Not Disrupt the Translocation Activity Produced by TatC and Mutated TatA Subunits—The M1 and M2 mutated versions of TatA were expressed in the presence of TatB and TatC to ascertain whether they could still function solely as TatA. Constructs were generated by site-directed mutagenesis using pBAD-ABCs as a template. First, the expression levels of TatABC were analyzed, and the results are shown in Fig. 2A. TatABC subunits were produced by all of the constructs tested, although no TatC signal is detected in the membranes of MC4100 cells because the wild type protein has no Strep tag.

TorA export assays were used to assess the translocation activity of these mutants, and the results are shown in Fig. 2B. All of the mutants are able to translocate TorA into the periplasm, although the M1 and M2 mutations appear to result in a lowered export efficiency when compared with the pBAD-ABCs or MC4100 cells. It seems likely that TatB has a much higher affinity for TatC than do the M1 or M2 mutants, and the absence of a stable M1/M2-TatC complex supports this idea (see below). In turn, this suggests that the mutant TatAs are still able to function primarily (if not solely) as TatA subunits. However, we stress that we do not have data on the relative affinities of M1/M2/TatB for TatC under these circumstances, and further studies are required to characterize the TatC binding partner(s) in the translocons.

The Mutated TatA Subunits Form Apparently Normal Heterogeneous Tat Complexes—Although the modified TatA subunits are able to compensate for an absence of TatB functionally, we wanted to address how the components were interacting with respect to the formation of Tat complexes in the membrane. A recent study (17) showed the presence of two different Tat complexes when TatABC is expressed. A TatABC complex was shown to migrate as a discrete 370-kDa band in blue native gels, whereas TatA formed variously sized homooligomeric complexes that migrated over a range of 100–550 kDa. As these complexes are thought to assemble to form an active translocon in E. coli, we sought to determine whether expression of the mutated TatA + TatC complexes leads to the formation of similar complexes in the absence of TatB. Solubilized membranes were prepared from cells expressing TatC together with wild type TatA or the M1/M2 forms of TatA, and these were separated by blue native gel electrophoresis. The top panel of Fig. 3 shows an immunoblot with TatA antibodies, with typical ladders of TatA complexes visualized after expression of TatABC (pBAD-ABC lane). No bands are visible in the ΔABCDE sample, but the remaining lanes show the presence of apparently typical TatA patterns in membrane extracts of cells expressing the wild type and M1 or M2 forms of TatA with TatCs. The data obtained with cells expressing wild type TatA + TatC confirm that the presence of TatA ladders is independent of translocation activity, and the remaining data show that M1 and M2 form a typical range of TatA complexes.

The Mutated TatAC Constructs Are Unable to Form a Discrete 370-kDa Substrate-binding Complex—The lower panel of Fig. 3 shows an immunoblot of the same BN gel using antibodies against the Strep II tag to visualize TatC-containing complexes. Previous studies (19, 20) show that the TatB and TatC subunits initially bind incoming substrate molecules (19, 20), and we have assumed that the 370-kDa TatABC complex carries out this role. Surprisingly, however, no distinct TatC-containing bands were observed when either wild type TatA + TatC or any of the mutated TatA + TatC subunits were expressed under the same conditions. As immunoblots show that TatC is expressed at similar levels in cells expressing wild type TatABC, A + Cs, M1 + Cs, and M2 + Cs (see Fig. 1B), this suggests that either TatC cannot form the same 370-kDa com-
plex with the M1/M2 variants of TatA or that any complex formed is highly unstable.

A TatA-TatC Chimera Supports Tat-dependent Translocation—Although any TatABC-like complex is undetectable, a productive interaction between the M1/M2 mutants and TatC must be occurring, because the expression of wild type TatA or TatC alone is not sufficient for translocation function (13, 35). We set out to test whether these two components could still support translocation when they were covalently linked by creating a translational fusion. A similar fusion of the E. coli TatB and TatC subunits is active (15), and our rationale was that linking the mutated TatA variants with TatC may stabilize the complex. We expressed a translational fusion of the tatA and tatC genes, TatACfus, under the control of the arabinose-inducible pBAD promoter, with the C terminus of TatA separated from TatC by a spacer region of 10 asparagine residues and a factor Xa protease cleavage site (IEGR). Similar constructs containing the M1 and M2 mutations were also prepared. The TatACfus protein includes 372 residues and is predicted to span the membrane seven times with the N terminus located in the periplasm and the C terminus located in the cytoplasm.

To examine the expression levels of the fusion proteins, membranes were prepared from pBAD-ABCs cells expressing the different plasmids. Fig. 4 shows these samples after immunoblotting with antibodies against the three Tat components. The individual Tat components are present in the pBAD-ABCs sample (although TatA antibodies recognize a second minor protein running more slowly than TatA). The remaining lanes contain samples from cells expressing a fusion of wild type TatA and TatC (Fig. 4A, TatACfus lane) or a mutated TatA and wild type TatC (M1Cfus, M2Cfus). The fusion proteins are expressed at similar levels, and no mature size TatA or TatC is detectable, indicating that none are proteolytically cleaved within the linker region to any detectable extent.

The translocation activities of the fusions were tested using the TorA activity assay, and surprisingly, M1Cfus and M2Cfus are active in Tat-dependent translocation (Fig. 5). The fusion between the wild type subunits (ACfus) is unable to export TorA, indicating that the substitutions in TatA are again responsible for translocation function.

TatACfus Forms Several Different Sized Complexes Containing TatA and TatC—To establish the nature of the functional translocon being formed by M1Cfus and M2Cfus, and to char-
TatA-TatC Containing Tat Complexes

FIGURE 5. The presence of excess free wild type TatA inhibits the translocation of TorA by M1 + Cs, M2 + Cs, M1Cs, and M2Cs. A, ABCDE, ABCDE containing pBAD-ABCs, or ABCDE containing pBAD-A + A + Cs or pBAD-A + ACfus, wild type, or with the M1 or M2 mutations in TatA as denoted were grown for 31/2 h at 37 °C in the presence of 50 μM arabinose, and subcellular fractions were prepared as described under “Experimental Procedures." A, membrane samples were immunoblotted using antibodies to TatA, TatB, or the Strep II tag. B, cytoplasm (C) and periplasm (P) fractions were electrophoresed on native polyacrylamide gels that were subsequently stained for TMAO reductase (TorA) activity. TorA, mature TMAO reductase; TorA*, more slowly migrating form that accumulates in the cytoplasm.

characterize the complexes generated by expression of the constructs, membranes were prepared and analyzed using BN-PAGE and immunoblotting with TatA and Strep II tag antibodies. The control lanes in Fig. 4B again show that in the absence of Tat components no bands are detected (ΔABCDE sample), whereas characteristic TatA ladders and a distinct TatABC complex of 370 kDa are observed when pBAD-ABC is expressed. However, the fusion proteins do not form complexes that resemble either the wild type complexes or those found after expression of the individual subunits. Approximately six bands are immunodetected with the Tat-specific antibodies, and these complexes appear to contain both TatA and TatC as they have the same mobility on each immunoblot. None of these complexes corresponds in size with the 370-kDa TatBC-containing complex or with any of the TatA homo-oligomers. It seems probable that these complexes result from interaction between TatA subunits, although the presence of attached TatC has made the difference in size between these complexes larger. The mass of each of the complexes was calculated as described previously (17) (calculation not shown). The smallest band has an apparent mass of 107 kDa, and thereafter the bands increase in increments between 27 and 55 kDa in mass with an average size of 44.6 kDa. Given that the mass of TatACfus is 38.5 kDa, the smallest complex seems to contain three TatACfus proteins with each larger band having only one more TatACfus protein added. A great deal of smearing is also evident with all of the fusion proteins, suggesting that they form a range of unphysiological complexes.

Interestingly, the ability to translocate Tat substrates does not appear to have any bearing on the type of complexes formed, as the same bands are observed with M1Cs and M2Cs, which can export TorA, and with the wild type ACfus, which cannot. The different TatA substitutions thus affect whether a complex has Tat function, but they have no impact on the structural conformation that the subunits assume.

The Presence of Excess Free Wild Type TatA Impedes Tat-dependent Export When Co-expressed with M1 and M2—We also investigated the effect of co-expressing wild type TatA with the mutated forms of both the individual TatA subunits (TatA + TatC) and the same subunits fused to TatC (TatACfus). The aim was to test whether the modified TatA subunits could function only as a TatB-like protein when wild type TatA is available. Constructs were generated for the co-expression of the modified and wild type proteins under the control of the same arabinose-inducible pBAD promoter. Membranes were prepared from cells containing each of the constructs to assess the expression levels of TatA and TatC, and the results are shown in Fig. 5A. The new constructs were tested alongside other constructs generated in this study to confirm that the protein expression levels were comparable. Expression of free wild type TatA plus mutant M1 + TatA is denoted as "A + M1 + Cs" and so on. The right-hand section of Fig. 5A represents cells expressing the fusion proteins ± free wild type TatA (e.g. A + M1Cfus, M1Cfus, etc). Membranes prepared from cells expressing additional wild type TatA, plus wild type TatAC, are denoted "A + A + Cs."

Fig. 5A shows that cells expressing A + M1 + Cs and A + M2 + Cs contain TatA, although it is not possible to distinguish between the modified and wild type versions. It is highly likely that both are being expressed, as TatA is situated before TatC in the plasmids, and TatC is visualized with all of these constructs. Membranes prepared from cells expressing A + ACfus, A + M1Cfus, and A + M2Cs contained the fusion protein, detected with both TatA and Strep tag antibodies, and individual wild type TatA subunits.

The ability of these cells to export TorA is analyzed in Fig. 5B. When additional wild type TatA is expressed with wild type TatA + TatC (A + A + Cs), no TorA activity is detected in the periplasmic fraction; this is as expected because TatA + TatC alone (A + Cs) could not support export. As shown above, M1 + Cs and M2 + Cs are able to translocate Tat substrates because a proportion of TorA activity was located in the periplasm (upper panel of Fig. 5B). However, the simultaneous expression of excess wild type TatA results in a complete block in export, as all of the TorA activity is present in the cytoplasm of cells expressing A + M1 + Cs and A + M2 + Cs. Apparently the expression of wild type and modified TatA within the same cell prevents the altered version from acting as a TatB-like protein.
**TatA-TatC Containing Tat Complexes**

Analysis of the fusion constructs shows a similar pattern (lower panel of Fig. 5B). M1Cfus and M2Cfus are able to translocate TorA, with significant TorA activity detected in the periplasm, whereas cells expressing A + M1Cfus and A + M2Cfus contain only cytoplasmic TorA activity. Again, the presence of free wild type TatA interferes with the ability of these fusion proteins to form a functional translocase.

Finally, we investigated the converse situation, in which a "wild type" TatACfus protein is expressed together with the mutated "free" TatA subunits, by expressing constructs where the wild type tatACfus was encoded after a single copy of either the M1 or M2 versions of tatA. The levels of TatACfus and TatA are shown in Fig. 5A (M1 + ACfus and M2 + ACfus lanes). Both constructs express the fusion well, although only a very faint band of TatA was immunodetected in membranes prepared from cells expressing M1 + ACfus. Fig. 5B shows that M1 + ACfus and M2 + ACfus are both able to support Tat-dependent export, as TorA activity is located in the periplasmic fraction of cells containing these plasmids. Even though wild type TatACfus alone cannot translocate TorA, the addition of the M1 and M2 proteins allows export of TorA to the periplasm. The ability of mutant TatA to support translocation under these conditions suggests that it may bind to TatC and effectively displace the wild type TatA from the critical TatB location.

**DISCUSSION**

The mechanism of the Tat system is poorly understood, but a variety of structural and functional studies has led to a model in which substrates initially bind to a 370-kDa TatABC complex (specifically, to the TatBC subunits), after which a homo-oligomeric TatA complex is recruited to form the full translocation system (15, 17–20, 36). Notably, TatA and TatB carry out completely different roles despite exhibiting significant sequence and structural similarity. The vast majority of Gram-positive organisms contain only tatAC genes, suggesting that the TatA subunit may fulfill both of the roles that are separately allocated to TatA and TatB in Gram-negative bacteria (12). Recent mutagenesis experiments on the E. coli Tat system appear to validate this argument by generating mutations in TatA that enable the system to work in the absence of TatB (25). Given the current interest in the roles of TatA and TatB, we considered it important to test whether these mutant TatA forms do actually carry out dual roles in the absence of TatB.

In the study of Blaudeck et al. (25), selection for anaerobic growth in the presence of TMAO resulted in the identification of TatA mutant proteins that must have gained the ability to translocate TorA. Because this notion was not directly addressed at the protein level, we have now extended this area of investigation and shown that the mutant TatA forms M1 and M2 are indeed capable of exporting reasonable amounts of the native TorA substrate despite the absence of TatB. This is an important point because TorA is a large protein (a dimer of 86-kDa subunits) containing a complex molybdopterin cofactor; hence the data confirm that all aspects of the Tat export pathway are functional, including synthesis, cofactor insertion, and translocation into the periplasm. The TorA export assay is only semi-quantitative, but the periplasmic band is usually less intense than the cytoplasmic, nonexported form. This, together with the findings described previously for the TorA-MalE hybrid precursor (25), suggests that the mutant forms of TatA are not as efficient as TatABC in supporting protein export.

We have used BN gels to characterize Tat complexes in these cells, and the data are striking; the characteristic ladder of TatABC complexes is clearly detectable, but we do not observe any distinct TatC-containing complexes in cells expressing free M1/M2 + TatC. Instead, a smear of bands is apparent, and this is clear evidence that the mutant TatA forms do not simply mimic TatB and readily assemble into stable "TatABC"-type core complexes. One possibility is that the complex is formed but unstable, and one of the reasons for generating TatA-TatC fusion proteins was to test whether these fusion proteins may form more stable complexes. However, these fusion proteins run as numerous bands on BN gels, and the regular spacing of the bands suggests that the fusion proteins are being assembled because of TatA-TatA interactions rather than forming substrate-binding complexes analogous to the characteristically discrete TatABC complex. Although the mutant TatA variants can support translocation activity in the absence of TatB, presumably by taking its place in the translocation system, these gross effects on the TatABC complex indicate that they cannot fully substitute for all the properties of TatB.

The other interesting feature of the M1-TatC and M2-TatC fusions is that they do support export of TorA at efficiencies that are close to those exhibited by the separate proteins. This observation is unexpected and, taken in conjunction with the absence of a detectable TatABC-like complex, has implications that have to be considered carefully. The key point is that translocation is occurring despite a complete absence of detectable TatABC-type or, in the case of the fusion proteins, TatA complexes. If these complexes are indeed integral elements of the translocation apparatus, a simultaneous lack of both (or at the very least, a huge reduction in the levels of both complexes) would be predicted to have a massive effect on translocation activity. Our results therefore raise the possibility that one or both of these recognized E. coli Tat complexes may not play a critical role in the translocation process. Whether or not this is the case, the data demonstrate that the active translocation complex may be formed from a transient assembly of complexes that are themselves undergoing dynamic changes in composition. However, a key point is that the translocase may be formed from complexes that have yet to be characterized in isolation.

Finally, it is interesting that the presence of wild type TatA is so inhibitory to the translocation supported by M1/M2 + TatC. One possibility is that the two forms of TatA are simply incompatible within the TatA complex (it may be formed, but unable to execute its function properly). Alternatively, M1 and M2 may have an abnormally high affinity for wild type TatA, such that insufficient M1/M2 are available to interact with TatC. Further work is required to unravel the precise effects, but this experimental setup may be useful for studying the key protein-protein interactions in this system.
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