Recumbent Expression of \textit{tatABC} and \textit{tatAC} Results in the Formation of Interacting Cytoplasmic TatA Tubes in \textit{Escherichia coli}\textsuperscript{S}

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The twin-arginine translocation (Tat) system of bacteria and plant plastids serves to translocate folded proteins across energized biological membranes. In \textit{Escherichia coli}, the three components TatA, TatB, and TatC mediate this membrane passage. Here we demonstrate that TatA can assemble to form clusters of tub-like structures in vivo. While the presence of TatC is essential for their formation, TatB is not required. The TatA tubes have uniform outer and inner diameters of about 11.5 nm and 6.7 nm, respectively. They align to form a crystalline-like structure in which each tube is surrounded by six TatA tubes. The tube structures become easily detectable even at only a 15-fold overexpression of the \textit{tatABC} genes. The TatA tubes could also be visualized by fluorescence when untagged TatA was mixed with low amounts of TatA-GFP. The structures were often found in contact with the cell poles. Because TatC is most likely polar in \textit{E. coli}, as demonstrated by a RR-dependent targeting of translocation-incompatible Tat substrates to the cell poles, and because TatC is required for the formation of aligned TatA tubes, it is proposed that the TatA tubes are initiated at polarly localized TatC.

Folded proteins can be transported across membranes by the twin-arginine translocation (Tat)\textsuperscript{3} system (1). The mechanism of this transport is unknown but must be quite unusual, because the transported proteins can be even larger in diameter than the membrane they are transported across (2). The minimal Tat translocon is composed of two components, TatA and TatC (3, 4). Proteobacteria and thylakoids require a third component for efficient translocation, TatB, which associates with TatC to form a TatBC complex (1). Because Tat substrates bind tightly only to TatBC complexes, and because a covalent cross-link to TatC still allows translocation, it is believed that TatC is the motor for the translocation process (5). TatA, however, also plays an essential role, most likely by allowing the membrane passage of the substrate, which is moved by TatC across the membrane (6). TatA is also the most abundant Tat component in \textit{Escherichia coli} (7). In the chloroplast system, the association of TatA with the TatBC complex occurs strictly after substrate binding (6), whereas TatA of Gram-positive bacteria has been detected inside the cytoplasm, where it has a high affinity for Tat substrates that might be targeted by TatA to the membranes (8–10). TatA of the \textit{E. coli} Tat system is known to form homooligomeric complexes (11). It has been purified from detergent-solubilized membrane fractions of strains overexpressing the \textit{tatABC} genes (12). The TatA complexes identified in that study varied in diameter and formed a ladder in blue native polyacrylamide gel electrophoresis (BN-PAGE) analyses. The variable sizes of these TatA complexes suggest the existence of some higher order homooligomeric TatA structure, which might have been disrupted by detergent treatment. We therefore analyzed strains overexpressing \textit{tatABC}, \textit{tatAB}, \textit{tatAC}, or \textit{tata} by transmission electron microscopy after high pressure freeze-fixation/freeze-substitution to improve the preservation of ultrastructural details (13, 14). We found that TatA is capable of forming homomultimeric tubes inside the cytoplasm, which can interact with one another in the presence of TatC to form quasi-crystalline structures. \textit{E. coli} TatA has therefore a previously unrecognized capacity to extend in three dimensions. Because these associations can sediment with membranes simply due to their size, the proposed strict membrane integral localization of \textit{E. coli} TatA has to be questioned. The Tat systems from Gram-positive and Gram-negative bacteria thus appear to be more similar than previously thought.

**Materials and Methods**

**Strains and Growth Conditions**—\textit{E. coli} MC4100 (15) or its \textit{tatABCDE}-deficient derivative DADE (16), was used for physiological studies, and \textit{E. coli} XL1-Blue Mfr\textsuperscript{*} Kan (Stratagene) was used for cloning. The bacteria were grown aerobically at 37 °C on LB medium (1% (w/v) tryptone, 1% (w/v) NaCl, 0.5%
**Plasmids and Genetic Methods**—The tatABC genes were expressed from pABS-tatABC (17), and selected tat genes were expressed from pABS-tatAC, pABS-tatAB, or pABS-tatA (18). A tatA-gfp fusion was expressed from pBAD-tata-gfp (17). A lower expression level of the tatABC genes was achieved with the vector pRK-tatABC (19).

For analyses of subcellular targeting of Tat substrates, we used a high potential iron-sulfur protein (HiPIP) Tat signal peptide fusion to five unfolded FG repeats (FG5) from Nsp1p (18). Construct expression vectors for C-terminal green fluorescent protein (GFP) fusions of RR-(FG5) or KK-(FG5), and TatA was expressed from pABS-tatA-H6 (19), or TatA-F39K-F (5’-GCT GTC GTT TTT ACC AAA AAG GCA ATG AGC GAT GAT G-3’), the fragment was cut with SacII and Xhol and cloned into the corresponding sites of pBW-R5 or pBW-R5-KK, resulting in pBW-R5-gfp and pBW-R5-gfp-KK, respectively. The tatA expression vector pBW-tatA-H6 was constructed by amplification of tatA with pABS-tatABC as template, using the primers tatA-Ndel-F (5’-GAA CAC ATA TGG GTG GTA TCA GTA TTT GCC-3’), restriction with NdeI/BamHI and ligation into the corresponding sites of pBW-R5 or pBW-R5-KK, and TatA was resuspended in 6 ml of the above mentioned buffer and loaded on a 1 ml nickel-chelate matrix column (Qiagen, Hilden, Germany). The column was washed with 15 volumes of buffer containing 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 20 mM imidazole, and 10% glycerol, and TatA was subsequently eluted in buffer containing 20 mM Tris-HCl pH 8.0, 50 mM NaCl, and 250 mM imidazole.

**Electron Microscopy Methods**—Bacteria growing in the exponential phase were concentrated by filtration, transferred into aluminum planchets (cavity 2-mm diameter, 0.3-mm depth) and frozen under high pressure with an HPM 010 apparatus (BALT, Liechtenstein). The cells were cryosubstituted with 0.25% (w/v) glutaraldehyde (Sigma) and 0.1% (w/v) uranyl acetate (Chemapol, Czech Republic) in acetone at −80 °C for 4 days using special cryosubstitution equipment (FSU, BAL-TEC). Thereafter, the temperature was increased up to −20 °C. Subsequently the samples were infiltrated stepwise with Lowicryl (HM20; Polysciences Europe, Eppelheim, Germany) and polymerized with UV light (24 h at −20 °C and 24 hours without cooling).

Ultrathin sections of 80-nm thickness were transferred onto nickel grids and stained with uranyl acetate and lead citrate in an EM-Stain apparatus (Leica, Bensheim, Germany). For immunogold labeling, ultrathin sections were treated with a polyclonal anti-TatA antibody and a secondary goat anti-rabbit IgG conjugated with 10-nm gold particles (Clontech Takara Bio Europe, St. Germain, France) prior to staining. Negatively stained samples were prepared by spreading 10 μl of the dispersion onto a Cu grid coated with a Formvar-film. After 1 min of adsorption excess liquid was blotted off with filter paper and 5 μl of 1% aqueous uranyl acetate were placed on the grid, drained off after 1 min and dried. Both ultrathin sections and negatively stained samples were observed with an EM 900 transmission electron microscope (Carl Zeiss SMT, Oberkochen, Germany). Micrographs were taken with a Variospeed SSCCD SM-1k-120 camera (TRS, Moorweitz, Germany). The software used for taking, storing, and analyzing the pictures (ITEM) was provided by Olympus Soft Imaging Solutions, Münster, Germany.

**Confocal Laser Scanning Microscopy**—For microscopical analysis, exponentially growing cultures were analyzed in LB medium without concentration or dilution of the sample. Strains containing pBAD-derivatives were grown for 3 h in the presence of l-arabinose at the concentrations indicated. To
immobilize cells, slides were thin-coated with 2% (w/v) agarose in LB medium. Laser scanning confocal data were acquired using a Zeiss LSM510 META mounted on an Axiovert 200M with a Zeiss water immersion C-Apochromat objective lens (63×/NA1.2 W). Excitation was at 488 nm, and emission was detected between 505 nm and 530 nm. Cells were visualized by differential interference contrast (DIC), using Nomarski optics unless otherwise indicated. Micrographs were captured with Zeiss LSM software.

RESULTS

E. coli TatA Can Form Ordered Tube Structures—TatA homologs are found in all known bacterial Tat systems (25). TatA is believed to function as a targeting factor in Gram-positive bacteria (8–10, 26). Solubilized TatA from E. coli membrane fractions migrates as a ladder of modular homomultimeric assemblies in BN-PAGE (11). Purified, detergent-treated E. coli TatA has been shown to form ring-like structures, prompting the well-recognized hypothesis that TatA may form a channel for the passage of Tat substrates (12).

We considered the possibility that the multitude of different TatA complexes might result from disassembly or reorganization of much larger complexes that could possibly be detectable by electron microscopy of cells after high pressure freeze-fixation (HPF), a method that can preserve sensitive structures within the cells. As the previously analyzed TatA complexes were purified from strains highly overexpressing the tatABC genes, we started our analyses with a strain that overexpresses the tatABC genes about 60-fold from a p15 origin vector. The cells of this strain often contained remarkably large regions of parallel structures, which were tightly associated in a very ordered manner (Fig. 1, A1). These structures were readily detectable in about half of the cell population, even though we looked only at thin sections of 80 nm, which cannot visualize the complete cell interior. The data therefore suggested that the structures were present in most, if not all, cells. As shown below, the observed parallel lines (Fig. 1, A1) are caused by tube-like structures that are cut in the longitudinal direction (inset of Fig. 1, A1). Albeit less abundant, the same structures were also detectable with the low copy pRK-tatABC vector system that confers only a 15–20-fold increase in the amount of the Tat components (Fig. 1C).

FIGURE 1. Detection of TatA tube structures within cells by electron microscopy. A, TatA tubes as formed in strain MC4100/pABS-tatABC, shown in a longitudinal cut (A1) or in a cross-section (A2). The insets schematically visualize how the structures are cut in the respective micrograph. B, immunogold labeling of TatA in DADE/pABS-tatABC. C, TatA tubes as formed in strain MC4100/pRK-tatABC, cut in longitudinal direction. D, TatA tubes in strain DADE/pABS-tatAC cut in perpendicular direction. E, micrograph of a representative cell from DADE/pABS-tatA. F, micrograph of a representative cell from DADE/pABS-tatAB. G and H, comparison of cross-sections of tube assemblies from DADE/pABS-tatABC (G) and DADE/pABS-tatAC (H). J1, TatA tubes as released from a broken cell of DADE/pABS-tatAC. J2, the same tubes at lower magnification, showing also the broken cell. Arrows indicate interconnections between aligned tubes. Arrowheads point to the central mass that is detected in the tubes in the absence of TatB. Bars, 50 nm.
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We also detected cross sections of these tube arrangements (Fig. 1, A2) in which a very regular assembly of tubes was observed with a central tube interacting with six surrounding tubes (inset of Fig. 1, A2). This 6-fold symmetry was observed in all detected tube assemblies, making it likely that the units that form these tubes exhibit a 6-fold symmetry, too. Notably, spike-like connections between the tubes were often visible, suggesting some direct interactions between the tubes (see arrows in Fig. 1, A2 and D).

The tube structures could have been formed by either of the overproduced TatA, TatB, or TatC components. We therefore immunogold-labeled the thin sections using primary antibodies against TatA, TatB, or TatC. Only TatA was detectable in the structures (Fig. 1B). Because TatA seemed to be the main constituent of the tube structures, we considered that the presence of TatB or TatC might not be necessary at all for the formation of aligned tubes. We therefore analyzed cells that overproduced TatA only or in combination with either TatB or TatC in the complete absence of all other Tat components.

When TatB was lacking, the tube structures were formed just as observed previously (Fig. 1, D and H). Obviously, TatB is not required for these structures to form. However, we noted that all tubes in the strain overproducing only TatA and TatC contained a single area of high electron density in their center (compare Fig. 1, H with G). Because the TatA tubes from strains co-overproducing TatA, TatB, and TatC were hollow, this suggests some influence of TatB on the tube structure. TatB may therefore either influence the conformation of TatA in the tubes, or prevent the inclusion of other proteins in these structures.

To ascertain the size of the tubes we measured the inside and outside diameters of 114 and 145 tubes, respectively. The mean inner diameter was 6.7 ± 0.8 nm and the mean outer diameter was 11.5 ± 1.2 nm.

In cells that were devoid of TatC, TatD, and TatE, but overproduced TatA and TatB (Fig. 1F), no ordered structures could be found. Instead, some shaded areas were present in these cells, indicating some sort of random aggregation. The same was observed with cells overproducing TatA only (Fig. 1E). It thus seems that TatA needs TatC to form detectable amounts of ordered structures.

In one sample, a disrupted cell (Fig. 1, J2) offered us a unique glimpse of the tubes in a cell-free situation, showing that the structures indeed consist of individual tubes (Fig. 1, J1). Interactions between tubes were disrupted upon cell lysis, and therefore they seem not to be very strong. As the tubes were released from a cell that overexpressed tatA and tatC, the tubes contained a continuous central thread (arrowhead in Fig. 1, J1), which we believe corresponds to the central dot-like electron density visible in the cross sections described above (arrowhead in Fig. 1H).

Fluorescence Microscopy Localization of the TatA Tubes—While electron microscopy is the technique of choice for obtaining high resolution pictures, it has the disadvantage of showing only a thin section of the cell. To find out more about the actual size and arrangement of the TatA tubes we attempted to visualize them by fluorescence microscopy using TatA fusions to the GFP. In the past we already analyzed the fluorescence in cells that produced only GFP-fused TatA together with native TatB and TatC, and we detected the fusion protein only at the periphery of the cell. This indicated that TatA is most likely membrane-associated, but not organized in larger cytoplasmic assemblies (17). Apparently, the fused GFP inhibited the interactions that lead to the formation of aligned tubes. This is not surprising, considering the size of GFP (near 30 kDa), which is considerably larger than that of TatA (near 10 kDa). Most likely GFP sterically hinders tube formation or tube alignment.

To circumvent this apparent inhibitory effect of GFP, we produced low amounts of TatA-GFP in a strain expressing the native tatABC genes from a compatible plasmid. To rule out any unspecific aggregation of the TatA-GFP fusion protein, we also analyzed a strain that produced TatA-GFP from the same arabinose-inducible vector in the complete absence of the other Tat components (Fig. 1, A and F). TatA was located peripherally in or at the membrane, which confirms our previous results on TatA-GFP localization (17). The fluorescence distribution changed dramatically as soon as we overexpressed the tatABC genes together with tatA-gfp. Not all cells showed fluorescence, but TatA-GFP was now detected in long fluorescent filaments that became visible in many cells (Fig. 2, B–E). Apparently, small amounts of TatA-GFP were incorporated into the TatA tubes. The fluorescent filaments often started at the cell poles, reaching far into the cell (Fig. 2, C and D). We even found very long structures that seemingly spanned the cell from one pole to the other (Fig. 2B). In all pictures the concentration of fluorescent TatA-GFP can be seen at the cell poles, whereas these polar dots were very rare in the negative control synthesizing TatA-GFP only (Fig. 2A).

Targeting of a Tat Substrate to Tat Translocons at the Cell Poles—Another way of localizing the Tat translocon is to track the whereabouts of Tat substrates during transport. One problem of this approach is that Tat substrates diffuse freely within the periplasm after translocation and thus do not necessarily indicate the position of the translocon. We solved this problem by using a fusion protein consisting of the Tat signal peptide from HiPIP fused to five natively unfolded FG repeats of the Nsp1p yeast nuclear pore protein and a C-terminal GFP. The FG repeats of this construct, named RR-(FG5)-GFP, thus serve as an unstructured linker peptide which connects the signal peptide to a folded GFP domain. The RR-(FG5) construct without GFP has recently been demonstrated to be Tat-dependently translocated (20). We now found that an additional folded GFP domain at the C terminus completely blocked translocation (Fig. 2F). The block of translocation by the GFP fusion is similar to earlier observations with other linker-containing fusion proteins (27). We analyzed also a RR>KK variant of the substrate. This exchange within the twin-arginine motif has previously been shown to block Tat transport of HiPIP and an interaction of the Tat system with a HiPIP signal peptide–PhoA fusion protein (19, 28). As expected, the KK-(FG5)-GFP was not translocated. We detected precursor of RR-(FG5)-GFP and KK-(FG5)-GFP in the membrane fraction, which is due to the earlier reported Tat-independent membrane interaction of this and other signal peptides (19, 29, 30).
When we compared the localization of RR-(FG5)-GFP with that of the KK-(FG5)-GFP negative control, we found small, distinct, and mainly polar spots with the RR-(FG5)-GFP construct (Fig. 2G), whereas we observed a uniform cytoplasmic distribution of (FG5)-GFP with the KK signal peptide (Fig. 2H). The fluorescent RR-(FG5)-GFP spots were not aggregates, as no inclusion bodies could be found by differential centrifugation (no sedimentation at 1.500 × g and 15.000 × g; Fig. 2K), by electron microscopy of cryo-fixed cells, or by fluorescence microscopy after cell disruption (data not shown).

The fluorescent foci indicate the position of active substrate-binding translocons for three reasons: (1) They depend on the presence of the twin-arginines in the signal peptide; (2) we had to overexpress the tatABC genes in this experiment to increase translocon-bound substrate fluorescence to levels higher than the background produced by unbound substrate; and (3) strains that overexpress tatAC alone, and thus lack TatB for the formation of an active translocon, did not show RR-(FG5)-GFP foci (see supplemental Fig. S1).

As the translocation is blocked with the substrate RR-(FG5)-GFP, it was possible that the fluorescent foci formed by this construct represent some stable translocation intermediate that accumulates in the membrane near the translocon. However, this is not the case. The spots are transient and clearly depend on the energy of the cells, as they disappear within 10 min of treatment with the uncoupler CCCP (Fig. 2J). The spots vanish upon cell disruption and in vivo cross-linking approaches so far failed to trap this transient interaction, suggesting that the fluorescent RR-(FG5)-GFP foci are not the result of an accumulated transmembrane intermediate. Rather, the results suggest they derive from short-lived reversible substrate-translocon interactions that might involve actively transporting translocons because energy and Tat functionality are required for these foci to form. As long as the overall transport mechanism is unclear and the in vivo interactions to Tat systems cannot be identified by cross-links in the bacterial system, it remains unclear at which stage exactly the GFP fusion affects transport.

Nonetheless, the data clearly show that under our experimental conditions there is a targeting of Tat substrates to a polar translocon component that recognizes the twin-arginine motif, which is TatC. A polarly localized TatC is also in full agreement with the polar origin of TatA structures described above, as TatC is essential for the formation of these structures.

**Self-assembly Reactions of TatA in the Absence of other Tat Components—In vivo**, TatA formed aligned tube structures only in the presence of TatC, and inclusions of no detectable in-gel fluorescence of RR-(FG5)-GFP after differential centrifugation of crude extract of strain MC4100/pABS-tatABC/pBW-RS-gfp. Samples were not heated prior to SDS-PAGE to conserve GFP fluorescence, which was scanned using a STORM 840 imaging system (GE Healthcare). The positions of RR-(FG5)-GFP precursor and degradation products are indicated. Precipitation forces are specified above the corresponding lanes. P, pellet; S, supernatant. Induction of P<sub>r</sub> was with 0.001% (w/v) L-arabinose in A–E and with 0.01% (w/v) L-rhamnose in G–K. Arrows indicate fluorescence-labeled TatA tubes C–E or the polar fluorescence of RR-(FG5)-GFP (G). Bars, 2 μm.
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ordered substructure when it was synthesized alone (Fig. 1). The detection of the TatA tubes was only possible due to their high degree of order. To determine whether TatA can form larger, unordered structures in the absence of TatC, which might escape detection in vivo, we decided to purify hexahistidine-tagged TatA from a strain devoid of all other Tat components. In fact, hexahistidine-tagged TatA has been used for purifications of TatA-containing Tat complexes in the past and thus is suitable for this purpose (12). We purified TatA-His6 without the use of detergents, as the above data suggested that a large portion of TatA apparently was not associated with the membrane. Furthermore, we avoided detergents to minimize disruptions or rearrangements of TatA structures. Notably, purified TatA-His6 showed a high degree of self-assembly, as evidenced by patterns of orthogonal or hexagonal clusters (Fig. 3, A and B). These patterns are rather fragmented and less ordered than the tube structures observed in vivo; however, they resemble the tube structures that are formed intracellularly when TatC is present. There were also many individual tubes detectable (Fig. 3C), which allowed the determination of the TatA tube outer diameter in these preparations, which was 17.7 ± 1.4 nm (n = 153). The relative to the in vivo data somewhat increased outer diameter of the individual TatA tubes was most likely a result of some flattening due to the drying step in the sample preparation. In agreement with the above data (Fig. 1), these results suggest that TatA has an intrinsic ability to self-assemble into large tube structures. In cells without TatC, these TatA structures end up in inclusion body-like aggregates (Fig. 1E). However, TatC appears to organize in vivo the self-assembly of TatA to yield the well-defined, ordered, and aligned TatA tubes (Fig. 1A).

Several Inactivating Mutations in TatA Affect the Formation of Aligned TatA Tubes—To examine the possible physiological relevance of the observed TatA structures, we analyzed the influence of inactivating point mutations in TatA on the formation of the tubes. In previous studies, the combination of random mutagenesis screens and targeted mutagenesis revealed several point mutations in TatA that led to a complete inactivation of Tat functionality (31). These were the mutations D31G and G33S, as well as several mutations of residue Phe-39 (namely F39A, F39K, F39Q, F39S, F39H), all of which are positioned in a postulated amphipathic helix. We generated the mutations D31G, G33S, and also two of the mutations at position Phe-39 (F39A and F39K) and analyzed their effect on the formation of TatA tubes as well as their effect on the translocation of the model Tat substrate DmsA-GFP. Whereas the mutation D31G had no significant effect on the formation of aligned TatA tubes, the mutations at positions Gly-33 and Phe-39 resulted in a complete absence of detectable TatA tubes (Fig. 4A). Importantly, the absence of TatA tubes was not due to instability and degradation of the mutated TatA, because TatA was stably produced in these strains, although the expression levels were somewhat lower than for wild-type TatA (Fig. 4B). Notably, the mutation with the lowest expression level, D31G, was the only one that showed the TatA structures, indicating that the influence on the tube formation was not due to a decreased level of synthesis.

As expected, the transport control showed that all of these mutations indeed drastically reduced (F39K) or completely
abolished (D31G, G33S, F39A) the translocation of the model Tat substrate DmsA<sub>33</sub>-GFP (Fig. 4A).

**DISCUSSION**

*TatA Forms Unusual Tube-like Assemblies—*The structural analyses of TatA complexes from *E. coli* have so far been carried out with solubilized membrane fractions of strains overexpressing the *tat* genes (12, 32). Ring- or pore-like structures have been described, and it has been proposed that these TatA structures represent the “Tat pore,” which allows the membrane passage of proteins. It was important to ask the following questions: (i) are these structures really derived from membrane integral complexes? and (ii) do they really represent structures that are unaltered during the purification procedure?

In our study we applied for the first time HPF and cryo-substitution for the detection of TatA complexes in *E. coli*. To our surprise, we readily detected quasi-crystalline arrangements of TatA tubes within the *E. coli* cytoplasm, which were formed upon overexpression of the *tatABC* genes. Although these tube arrangements were destroyed upon cell lysis, the TatA structures remained sufficiently large to sediment together with membranes during ultracentrifugation. This observation indicates that a membrane association of TatA cannot necessarily be concluded from the co-sedimentation with membranes. Moreover, our results show that the TatA structures are clearly tubes and not rings. The previous observation of ring-like structures can probably be explained by detergent treatment of the samples during the purification process (12, 32). The effect of these detergents was perhaps not only an extraction of TatA from the membrane bilayer, but also the disruption of larger TatA structures to smaller modules, which could no longer sediment at high centrifugal forces. This is also supported by our observation that TatA can be purified from membrane fractions without the use of any detergent and that this TatA also self-assembles (Fig. 3). Reported ring- or pore-like structures of purified TatA thus appear to be a result of detergent treatment and self-assembly reactions during purification. They probably do not originate from membrane-integral TatA complexes, but rather from much larger cytoplasmic structures.

*What is the Physiological Relevance of the TatA Tube-like Structures?—*The observation of TatA tubes inside the *E. coli* cytoplasm is puzzling: TatA interacts with the TatBC/Tat substrate ternary complex and initiates translocation (33, 34). In addition, because of the co-fractionation of TatA with membranes, which depended on the hydrophobic N-terminal region of the protein, it has been assumed that TatA must be an exclusively membrane-bound component of the Tat system (35). However, electron microscopy of TatA<sub>d</sub> from *Bacillus subtilis* revealed that a significant portion of this TatA was present in the cytoplasm where it was able to bind the Tat substrate PhoD (8, 26). The TatA<sub>d</sub>, which interacted with membranes, had substrate bound to it (9). Similarly, in *Streptomyces lividans* significant amounts of TatA were found to be soluble and this TatA had a high affinity for Tat substrates in the absence of membranes (10). Furthermore, purified TatA<sub>d</sub> from *B. subtilis* has already been shown to form short tube structures (9).

We show here that TatA complexes can stably exist also in the cytoplasm of *E. coli*. A population of TatA is certainly present at the cytoplasmic membrane and has been analyzed in several topology studies (35–37). Under normal growth conditions, the N and the C termini of TatA appear to be cytoplasmic (35, 37) and possibly a small portion of TatA is exposed to the periplasm at its hinge region (37). Our data presented in this study are in full agreement with these observations, as is the previously found halo-like fluorescence of TatA-GFP in *E. coli*, which indicated that TatA *per se* can interact with the cytoplasmic membrane (17). We know now that the GFP fusions influence the localization of TatA, as the production of TatA-GFP, together with TatB and TatC, does not result in the formation of the aligned tube structures, unless native TatA is co-produced (Fig. 2). TatA-GFP interacts preferentially with membranes, but this localization could not be confirmed so far with native TatA. The EM data we obtained with untagged and unfused, but over-produced, Tat components clearly demonstrate a cytoplasmic localization of significant TatA populations. The TatA-GFP fluorescence in the presence of larger amounts of unmodified TatA confirms these conclusions and visualizes the structures in whole cells. These data suggest that TatA does not require a membrane environment for stable folding and self interactions, although the membrane integral component TatC is essential for the alignment of the tubes that can be detected *in vivo*, and thus lipid contacts or small lipid contents of the TatA tubes cannot be excluded.

The need for TatC and the astonishingly highly ordered nature of the TatA tubes makes a physiological role for these structures likely. TatA has the intrinsic ability to multimerize (11, 35, 38). *In vivo*, TatC acts like an initiator for TatA tube assembly. This is underlined by the observation that most TatA structures are anchored at the poles where TatC is most likely localized (Fig. 2G). We believe that our evidence for a polar TatC localization is stronger than contradictory evidence obtained with GFP-tagged TatC variants (39), because we localized natural Tat translocons indirectly by using a translocation-incompatible GFP-fused substrate. The poles were labeled by this substrate in a twin-arginine-dependent manner, effectively ruling out any unspecific aggregation at the cell poles. As TatC is the only Tat component that directly recognizes the twin-arginine motif (5, 34), this suggests that the unmodified TatC was present at the cell poles.

TatA tubes become detectable under overproduction conditions when they form large aligned clusters. At wild type level of synthesis, TatA tubes are likely to be much shorter and less extensively aligned. As the formation of highly ordered TatA tubes is a fact that has to be explained somehow, one can imagine two distinct theoretical possibilities of how detectable TatA tube arrangements are formed:

(i) The “membrane-only hypothesis” is based on the idea that TatA normally forms a membrane integral complex that laterally interacts with TatC. Excess TatA modules may artificially assemble onto membrane-integral TatA, which could induce the observed filamentous assemblies. According to this hypothe-
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esis, TatA modules would normally exist only in the lipid environment. The tube formation in the cytoplasm would be a coincidental artifact, as would be the requirement for TatC, the influence of TatB, the obvious stability of structured TatA assemblies in a cytoplasmic environment, the interactions between the tubes, the uniform dimensions of the tubes in all cells, and the effect of inactivating point mutations in TatA on the tube structures.

(ii) The “dual localization hypothesis” on the other hand proposes that soluble TatA modules or short tubes exist alongside membrane-localized TatA. Interactions with membranes may be reversible, and most likely occur via TatC. TatA is also known to have a natural affinity for membrane surfaces (35). This affinity could play a major role in the transport process when TatA interacts with substrate-bound TatBC complexes. TatA modules might interact with other TatA modules or with TatC via the same interaction regions. This model would explain the TatC dependence of TatA tube formation, the influence of TatB on the structures, and also the well-defined dimensions and the stability of TatA structures.

The observed characteristics of the TatA tubes make the second model more plausible to us. It is also attractive because it questions the differences between the ideas for TatA localization and TatA-TatC interactions in Gram-positive and-negative bacteria. In both groups, short TatA tubes may function as targeting factors for substrates, and/or they could play a role in the mechanism of translocation after the binding to the other Tat components.

The symmetry of the tube alignments suggests that modules of TatA tubes consist of six or a multiple of six TatA subunits. Connections between the tubes point to a natural occurrence of these interactions. The requirement of TatC for the symmetric TatA tube arrangements may also point to a 6-fold symmetry of TatC. Perhaps the Tat translocons are not evenly distributed at the cell poles but there might be a number of small “translocon rafts” present in the cytoplasmic membrane. Such translocation “microdomains” are also known from the Sec system in Streptococcus pyogenes (40). In the case of the Tat system, these could serve as translocation foci consisting of a number of translocons, each with a central core made of TatC and TatB and possibly one or a number of short tubes consisting of TatA. Each translocon would have six neighboring translocons. The extended growth of the TatA tubes upon recombinant production could be the basis for the observed quasi-crystalline arrangements. The tight concentration of translocons at one spot could confer advantages to the cell, for example to facilitate “damage control” of proton leakage during transport. The interactions could also contribute to the overall rigidity of the translocons in the membrane, thereby supporting the passage of folded proteins.

Although there remains much to learn about the exact nature of these newly discovered Tat tubes and their role in the actual translocation process, we believe them to highlight fascinating and as yet unknown properties of the Tat system, and we hope that our work can contribute to a better understanding of this unique translocation mechanism.

Acknowledgments—We thank Jan R. Andersen and Gary Sawers for discussions and support.

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