VirB8 homolog TraE from plasmid pKM101 forms a hexameric ring structure and interacts with the VirB6 homolog TraD

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Type IV secretion systems (T4SSs) are multiprotein assemblies that translocate macromolecules across the cell envelope of bacteria. X-ray crystallographic and electron microscopy (EM) analyses have increasingly provided structural information on individual T4SS components and on the entire complex. As of now, relatively little information has been available on the exact localization of the inner membrane-bound T4SS components, notably the mostly periplasmic VirB8 protein and the very hydrophobic VirB6 protein. We show here that the membrane-bound, full-length version of the VirB8 homolog TraE from the plasmid pKM101 secretion system forms a high-molecular-mass complex that is distinct from the previously characterized periplasmic portion of the protein that forms dimers. Full-length TraE was extracted from the membranes with detergents, and analysis by size-exclusion chromatography, cross-linking, and size exclusion chromatography SEC) multilange light scattering (MALS) shows that it forms a high-molecular-mass complex. EM and small-angle X-ray scattering (SAXS) analysis demonstrate that full-length TraE forms a hexameric complex with a central pore. We also overproduced and purified the VirB6 homolog TraD and show by cross-linking, SEC, and EM that it binds to TraE. Our results suggest that TraE and TraD interact at the substrate translocation pore of the secretion system.

Significance

The overproduction and purification of membrane proteins are intrinsically difficult, making their analysis challenging. We purified the TraE membrane protein from a bacterial conjugation system that is involved in plasmid transfer. Our results suggest that this protein forms hexamers with a central pore, and we also show that it binds to the TraD protein. The structure of TraE is completely different from that of the previously characterized periplasmic domain. This has intriguing implications for the role of TraE and of its interaction partner TraD in substrate translocation across the bacterial cell envelope. This work makes an important contribution to understanding of the mechanism of plasmid transfer, contributing to the design of approaches to inhibit the spread of antibiotic resistance genes.

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Data deposition: The data reported in this paper have been deposited in the Small Angle Scattering Biological Databank, www.sasdb.org (accession no. 5ASD75).

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Since expression of membrane proteins can be a considerable challenge, we first optimized the conditions for overproduction of N-terminally His-tagged, full-length VirB8 homologs from Brucella suis (VirB8b), H. pylori (CagV), and plasmid pKM101 (TraE) (Fig. 1A). Since expression of traE resulted in the highest amount of overproduction, we pursued this protein and analyzed the efficacy of extraction from the membrane with six different detergents (Fig. 1B). Extraction with octyl glucose neopentyl glycol (OGNG) gave the highest yield and was used for membrane extraction, followed by purification via immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC) (27).

Cross-Linking and SEC-MALS Show That Full-Length TraE Forms a High-Molecular-Mass Complex. In our previous work, we have shown that the periplasmic domains of VirB8b and TraE form concentration-dependent dimers, and multimer formation can also be monitored by cross-linking (8, 10). To compare the multimeric state of full-length TraE relative to that of its periplasmic domain, we subjected equal amounts of the proteins to cross-linking with varying concentrations of the homo-bifunctional cross-linking agent disuccinimidyl suberate (DSS). The periplasmic domain primarily forms dimers (Fig. 2A), and low amounts of higher molecular-mass multimers are observed at higher protein concentrations (8). In contrast, when the full-length protein is incubated with DSS, dimers, as well as higher molecular-mass multimers, successively form and the monomeric protein diminishes, indicating high cross-linking efficacy (Fig. 2B). This result suggests that the quaternary structure of the full-length protein is quite distinct from that of the periplasmic portion. To obtain quantitative information, we further characterized TraE by SEC-MALS analysis, enabling us to calculate the absolute molecular masses of the TraE oligomer (207 kDa), of OGNG micelles (41 kDa) (SI Appendix, Fig. S1), and of the protein–detergent complex (248 kDa) (Fig. 2C). According to the molecular mass derived from MALS analysis, TraE may form hexamers or heptamers, and we next obtained direct insights into the structure of these complexes.

EM and SAXS Analyses Suggest That TraE Forms Hexamers. To obtain independent evidence for the quaternary structure of TraE, we analyzed purified detergent-solubilized TraE by negative-stain EM. We observe uniform ring-like particles of ~130 Å diameter, and the particles apparently have similar orientations (Fig. 3A).

Fig. 1. Overexpression and detergent solubilization of VirB8-like full-length proteins. (A) Western blot analysis with a His-tag–specific antiserum to test the overexpression of VirB8-like proteins using the indicated concentrations of the expression inducer IPTG: Brucella (VirB8b; 30 °C, 6 h), Helicobacter (CagV; 30 °C, 6 h), and pKM101 (TraE; 18 °C, 16 h). (B) Western blot analysis with a His-tag–specific antiserum to test the solubilization of TraE in several detergents (CHAPS, DM, DMNG, LMNG, and OGNG). CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; DM, decyl maltoside; DMNG, decyl maltose neopentyl glycol; LMNG, lauryl maltose neopentyl glycol; OGNG, octyl glucose neopentyl glycol. Arrows indicate optimal conditions.

Fig. 2. Analysis of the oligomerization state of TraE. SDS/PAGE analysis of the purified periplasmic domain of TraE at 1 mg/mL (A) and of purified full-length TraE at 1 mg/mL (B) in the absence (0 mM) and presence of increasing concentrations (0.2–2.0 mM) of the cross-linking agent DSS. Proteins in the gels were stained with Coomassie blue dye, and arrows indicate higher molecular-weight complexes formed after cross-linking. (C) Elution profile of the TraE oligomer is shown with the molecular weight estimated by MALS. The molar masses corresponding to the total complex, the TraE oligomer, and the modifier (detergent micelle) throughout the elution peaks are shown.
EM Confirms That TraD and TraE Form a High-Molecular-Mass Complex. To obtain additional insights into the structure of the TraD–TraE complex, we conducted negative-staining EM analyses revealing that these proteins form a complex of smaller dimensions compared with the TraE complex (Fig. 6 and SI Appendix, Fig. S3). This result is consistent with the analysis by SEC and the overall asymmetrical shape of this complex (53 Å × 103 Å), comprising a core body with an elongated extension that is quite distinct from the symmetrical hexamer, with a diameter of 130 Å formed by TraE alone (Fig. 3A). To corroborate the

Fig. 4. SEC-SAXS analysis of TraE. (A) SEC profile of the TraE sample used for the inline SAXS experiment. mAU, arbitrary units. (B) Normalized pair distribution functions [P(R)] calculated automatically with AutoGNOM. (C) Fit of the theoretical scattering profile for the rigid body model (gray plot) with the experimental SAXS data (black line). A top view (D), side view (E), and down view (F) of the average molecular envelope calculated for TraE (Small Angle Scattering Biological Databank SASDB75) are shown. The approximate envelope dimensions are illustrated.
presence of TraD and TraE in the complex, we conducted immuno-EM analysis with TraE-specific antibodies. Hisc-specific beads, and gold bead-labeled secondary antibodies; negative-staining EM confirms the presence of both proteins in the complex (SI Appendix, Fig. S4).

**Discussion**

The results presented here provide insights into the structures of integral inner-membrane T4SS components. Until now, VirB8-like proteins were primarily considered as assembly factors, and this notion was based on the fact that they interact with many other T4SS components, mostly in a relatively transient fashion. Biochemical work showing these interactions was performed primarily with the purified periplasmic domains of the protein (8–10, 13, 28), but interactions of full-length VirB8 homologs were also shown using the bacterial and yeast two-hybrid systems (12, 14, 29–31). The fact that the quaternary structure of full-length TraE is very different from that of the periplasmic portion underlines a critical role of the N-terminal transmembrane helix and of the cytoplasmic domain for protein assembly and function. The role of these parts of the protein attracted relatively little attention until now, but studies with the bacterial two-hybrid system showed that they contribute to dimerization (29–31). The fact that purified full-length TraE assembles into a hexamer with a possible pore at the center was unexpected and suggests that the protein also plays a structural role in the T4SS. This notion is consistent with previous observations suggesting that the protein binds the translocated DNA substrate in Agrobacterium (24).

The exact localization of VirB8-like proteins in T4SS complexes is currently unknown, but the SAXS model proposed here enables us to dock the protein into the available structures obtained by X-ray crystallography and EM (6, 7, 32). The dimensions of the SAXS model, combined with knowledge on the localization of TraE at the inner membrane, enable docking into the T4SS structure to the top of the “arch” observed in the structure of the plasmid R388 T4SS5–10 (7) (SI Appendix, Fig. S5). Considering the available data on VirB8/TraE protein interactions with VirB4 and VirB6 homologs, this localization would be biologically significant and is consistent with the previously observed VirB8-VirB6-VirB4 cofractionation (9). The hexameric TraE structure may localize on top of VirB4-like proteins that were shown to be present in two copies of hexamers (7) (SI Appendix, Fig. S4). The observation of VirB8 hexamers and the docking of two copies on top of VirB4 complexes are also consistent with the experimentally determined stoichiometry of ~12.6 VirB8 molecules in the T4SS subcomplex from plasmid R388 T4SS5–10 (7). Previous work in the Agrobacterium system showed cross-linking of VirB8 to translocated DNA substrates, which suggested that VirB8 and VirB6 form a functional subcomplex that possibly constitutes the pore of the T4SS (24). We here present direct evidence for a complex between VirB8- and VirB6-like proteins, supporting the notion that they act together in substrate translocation. The TraE multimer is strikingly similar to the higher molecular-mass complex observed in the case of TraM; its homolog from the plasmid R64 conjugation system and complexes of similar dimensions were also observed in the case of the homolog DotI from the Legionella pneumophila T4SS (33). The SAXS and EM results presented here demonstrate that this protein forms a hexamer with a central pore, and this discovery has important implications for the mechanism of type IV secretion. In addition, docking of the TraE structure into the center of the structure of a T3SS (34) shows that the hexamer would fit the dimensions of these macromolecular transporters in a similar fashion, even if T3SSs are not evolutionarily related and do not contain VirB8 homologs (SI Appendix, Fig. S5B).

Based on the available data, we propose a model for the assembly of VirB8/TraE-like proteins at the inner membrane of gram-negative bacteria (SI Appendix, Fig. S6). VirB8-like proteins integrate into the inner membrane following the standard protein insertion pathway, followed by dimerization. The available X-ray structures of VirB8 from Brucella (17) and Agrobacterium (18) and of TraE from pKM101 (8) show alternative modes of dimerization. These may reflect alternative conformational states of the protein that assembles into a hexamer, possibly exposing different interfaces for interactions with other T4SS components (e.g., with the pilus components VirB2 and VirB5), and this
process may be coordinated via its interaction with VirB4 (9). The interactions with structural components such as VirB10 (11, 14) may link VirB8 complexes to the central substrate translocation pore that may be formed by VirB6 (13, 24). The TraD–TraE complex characterized here with biochemical methods and by EM substantiates the notion that these proteins interact, but it is smaller than the TraE hexamer alone. Therefore, the TraD–TraE complex likely represents an intermediate state for the assembly of these proteins at the T4SS core.

In the future, higher resolution structural studies of VirB6/TraE and of its complexes with VirB6 homologues and with other proteins (e.g., by X-ray crystallography or cryo-EM) are required to test this model and to establish the role of the versatile VirB8-like proteins and their complexes with VirB6 in T4SS assembly and function.

**Experimental Procedures**

**Strains, Plasmids, and DNA Manipulation.** The strains and plasmids used are described in SI Appendix, Table S1. The E. coli strain XL-1 Blue or DH5α was used as a host for cloning, and the E. coli strain BL21(DE3)star was used for VirB8, CagV, TraD, TraE, and TraD–TraE complex protein overproduction. Miniprep kits (Qiagen) were used to isolate plasmid DNA. Standard techniques were employed for the cloning, transformation, preparation, and restriction analysis of plasmid DNA from E. coli (35).

**Small-Scale Membrane Protein Expression and Solubilization Tests.** For protein overproduction, the E. coli strain BL21Star (DE3) carrying expression plasmids was grown under aerobic conditions at 37 °C in LB to exponential phase (OD600 of 0.4–0.8). For VirB8, CagV, and TraE, expression was induced by the addition of 0.1, 0.5, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG); temperatures at 18 °C, 25 °C, 30 °C, and 37 °C; and cultures left shaking for 1–1.5 h at 220 rpm. Cells were harvested by centrifugation at 12,000 × g for 10 min at 4 °C. Bacterial pellets were suspended in lysis buffer [50 mM Hepes (pH 8.0), 250 mM imidazole] containing 5 mM MgCl2, 1% Triton X-100, 1 mg/ml lysozyme, and one tablet of Complete Mini Protease Inhibitor mixture (Roche) and kept on ice for 1 h. To separate soluble and insoluble fractions, centrifugation at 32,500 × g for 30 min at 4 °C was done, and the supernatant was used for isolation of the membranes. Proteins in cell lysates were detected by Western blotting with an anti-His-tag antiserum (1:5,000 dilution) to detect His–VirB8 complex protein overproduction.

**Chemical Denaturation and E. coli Membrane Proteome.** To determine the VirB8 complexes with VirB6 homologues and with other proteins (e.g., by X-ray crystallography or cryo-EM) are required to test this model and to establish the role of the versatile VirB8-like proteins and their complexes with VirB6 in T4SS assembly and function.

**Protein–Detergent Complex Analysis.** The TraE–OGNG complex was analyzed by SEC-MALS with the use of an AKTAmicro system (GE Healthcare) coupled to a Dawn HELEOS II MALS detector and an OptiLab T-Rex online refraactive index detector (Wyatt Technology). The absolute molecular mass was calculated by analyzing the scattering data using the ASTRA analysis software package, version 6.16.5 (Wyatt Technology). Protein samples were separated on a Superdex 200 10×300 analytical SEC column (GE Healthcare) with a flow rate of 0.3 mL/min. BSA was used for calibration. A 0.1-mL sample of TraE at a concentration of 1 mg/mL was injected and eluted in 50 mM sodium phosphate buffer (pH 7.4), 300 mM NaCl, 40 mM imidazole, and 0.15% OGNG. The molecular masses of TraE and OGNG were determined by the dual detection method implemented in the conjugated analysis mode of the ASTRA analysis software. The refractive index increment of OGNG was calculated using the d/dc determination method developed by Wyatt Technology (SI Appendix, Fig. S1). The refractive index increments of TraE and OGNG used were 0.185 mL·g⁻¹ and 0.118 mL·g⁻¹, respectively. The extinction coefficient of TraE for UV detection at 280 nm was calculated from the amino acid sequence.

**Analysis of Homo-Oligomerization of TraE, Homo-Oligomerization of TraD, and Hetero-Oligomerization of TraE–TraD Complex with Cross-Linking.** Chemical cross-linking was performed with DSS (Pierce) as performed as described elsewhere (9). The cross-linking product formation for homo-oligomerization of TraE was monitored by SDS/PAGE and staining with Coomassie blue dye. For homo-oligomerization of TraD and hetero-oligomerization of TraD–TraE complex, the formation of cross-linking products was detected by Western blotting with an anti-His-tag antisemur (1:5,000 dilution) to detect His–TraE and with an anti-TraE antisemur (1:3,000 dilution), respectively.

**Negative-Stain EM, Image Analysis, and Gold Labeling.** Parlodion-supported and carbon-coated copper grids (SPI Supplies) were negatively glow-discharged (Leica Microsystems) before adsorbing 5 μL of SEC-purified sample at 2 ng/μL for 1 h and stained with 5 μL of freshly prepared 1.5% uranyl formate (Electron Microscopy Sciences) for 1 min. Samples were imaged at room temperature using an FEI Tecnai T12 transmission electron microscope (TEM) equipped with a tungsten filament and operated at 80 kV. Images were collected at defocus between 2 and 4 μm. Oligomerization of TraD and hetero-oligomerization of TraD–TraE complex was analyzed using the d/dc determination method developed by Wyatt Technology (SI Appendix, Table S1).

**Protein Data Bank (PDB)**
covering a scattering vector range \( q = 4\pi \sin(\theta)/\lambda \) from 0.008 to 0.8 \( \AA \). Data were analyzed, integrated, and averaged with RAW software package, version 1.0.1 (30). Buffer blanks were averaged and subtracted from the data. A linear Guinier fit plot was calculated using the RAW software. Guinier analysis and Rg estimation were performed in PRIMUS and confirmed by automatic analysis using AutoRG (39). The largest dimension of the molecule, \( D_{max} \), and \( V_p \) were calculated using GNOM (40). The pair distribution function \( P(R) \) and forward scattering \( I(0) \) were computed with AutoGNOM (41) and compared with those determined in Primusqt (39).

GASBOR (42) was used to reconstruct an ab initio protein structure by a chain-like ensemble of dummy residues, and a P6 symmetry was applied. Structural figures were prepared using PyMOL (43) and UCSF Chimera (44). To fit the SAXS envelope with X-ray and EM structures, the MultiFit add-on from UCSF Chimera was used (45), and, finally, a manual orientation of the X-ray structure was done. Data have been deposited in the Small Angle Scattering Biological Database (www.sasdb.org; SASDB75).

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