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Outer Membrane Translocation Arrest of the TcpA Pilin Subunit in *rfb* Mutants of *Vibrio cholerae* O1 Strain 569B

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The toxin-coregulated pilus (TCP) of *Vibrio cholerae* is a type 4-related fimbrial adhesin and a useful model for the study of type 4 pilus biogenesis and related bacterial macromolecular transport pathways. Transposon mutagenesis of the putative perosamine biosynthesis genes in the *rfb* operon of *V. cholerae* 569B eliminates lipopolysaccharide (LPS) O-antigen biosynthesis but also leads to a specific defect in TCP export. Localization of TcpA is made difficult by the hydrophobic nature of this bundle-forming pilin, which floats anomalously in sucrose density gradients, but the processed form of TcpA can be found in membrane and periplasmic fractions prepared from these strains. While TcpA cannot be detected by surface immunogold labelling in transmission electron microscope preparations, EDTA pretreatment facilitates immunofluorescent antibody labelling of whole cells, and ultrathin cryosectioning techniques confirm membrane and periplasmic accumulation of TcpA. Salt and detergent extraction, protease accessibility, and chemical cross-linking experiments suggest that although TcpA has not been assembled on the cell surface, subunit interactions are otherwise identical to those within TCP. In addition, TcpA-mediated fucose-resistant hemagglutination of murine erythrocytes is preserved in whole-cell lysates, suggesting that TcpA has obtained its mature conformation. These data localize a stage of type 4 pilin translocation to the outer membrane, at which stage export failure leads to the accumulation of pilin subunits in a configuration similar to that within the mature fiber. Possible candidates for the outer membrane defect are discussed.

Vibrio cholerae is a gram-negative pathogen of great significance in terms of its impact on global health, and it provides an important and well-studied example of an organism that secretes a variety of proteins. One such protein is TcpA, the major structural subunit of the toxin-coregulated pilus (TCP), a pilus adhesin of the type 4 class that is essential to pathogenesis (1, 12, 56) and is highly conserved in both *V. cholerae* O1 biotypes (14).

The cholera organism is useful as a vehicle for the study of type 4 pilin assembly because of the utility of its genetics, the clustering of the *tcp* genes, and the presence of related systems (18, 32). Homologies within type 4 pilin export pathways have been recognized for some time to extend into systems dedicated to the transport of substrates as diverse as proteins, naked DNA, and bacteriophage (13, 40). The short leader sequence first attributed to the type 4 pilins, and cleaved by the characteristic prepilin peptidase (19), is also seen in pseudopilin members of these nonpilin pathways (2, 4, 13, 40, 42). This peptidase may be shared between pilin and nonpilin systems (53), and processing of pseudopilin substrates has been shown to be essential for their function (2, 41).

The existing models of type 4 pilin assembly involve cytoplasmic membrane assembly of pilus (4, 53), but certain highly conserved outer membrane elements are also known to be essential (27), and the fate of type 4 pilin subunits after processing at the cytoplasmic membrane is unknown. While the peptidase, its substrates, and a cytoplasmic membrane-associated ATP binding protein are almost universal partners in pilin and nonpilin pathways (13, 40), the nature of other participating elements within the outer membrane is less clear. The best-studied and probably the most important class of protein is that exemplified by the pIV protein of f1 filamentous bacteriophage (44), the PilQ protein of *Pseudomonas aeruginosa* (27), and the PulD protein of *Klebsiella oxytoca* (3). Data from studies of pIV and PulD (11, 21, 44) suggest that these proteins probably form multimeric gated channels in the outer membrane, such as might be suitable for a variety of macromolecular traffic. In the absence of a *tcp* homolog, one must propose that a homolog exists outside the operon, that a functionally analogous protein exists which does not share amino acid sequence homology, or that there is an alternative mechanism altogether for TCP. Candidate homologs might be found in the related *eps* transport system, for example, which is responsible for export of the coregulated cholera toxin in *V. cholerae* (33). Within the *tcp* operon itself, however, *tcpC* and *tcpF* appear to encode, respectively, an outer membrane lipoprotein of unknown function and a possible porin-like protein that could conceivably participate in the formation of an analogous multimeric channel (20, 30, 37).

Misassembly of outer membrane multimers in lipopolysaccharide (LPS) mutants has been described (24, 48), and the significance of LPS to other transporters has been emphasized (61). Transposon-induced mutagenesis of the *rfb* genes encoding the putative perosamine biosynthesis pathway in *V. cholerae* 569B gives rise to a rough phenotype in which the LPS lacks O antigen (62). Preliminary observations suggested a TCP export-deficient phenotype in these strains (51). Subsequent studies have confirmed this, along with restoration of TCP assembly upon complementation of the *rfb* defect (16). In these strains, but not the isogenic wild-type (*wt*) parents, TcpA is detected in periplasmic fractions derived by simple differential centrifugation after lysozyme-EDTA treatment (16).

In this article, we show that stably accumulated TcpA pilin subunits in a 569B*rfb* mutant fail to translocate beyond the inner face of the outer envelope, we explore the physical con-

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text of the translocation-arrested state, and we propose a candidate protein in the outer membrane.

MATERIALS AND METHODS

Media, reagents, and antibodies. Restriction enzymes and T4 DNA ligase were obtained from Boehringer-Mannheim, and *Taq* polymerase was from Cetus-Perkin Elmer. [^{35}S]methionine was obtained from Amersham, and the cross-linkers dithio-*bis*-succimidyl propionate (DSP) and 3,3′-dithiobis(sulfonyl succinimidyl propionate) (DTSSP) were from Pierce Chemicals. Other chemicals and reagents, including trypsin and proteinase K, were obtained from Sigma.

Tc20.2 (mouse immunoglobulin G [IgG]), a monoclonal antibody to TcpA (31), was a generous gift from Gunhild Jonson and Jan Holmgren (University of Göteborg, Göteborg, Sweden). The polyclonal anti-569B-165 antisera to TcpA have been described previously (50). Polyclonal antisera raised against the cytoplasmic membrane protein, TcpT, have been described separately (15).

Bacterial strains and growth conditions. The strains used were *V. cholerae* O1 (classical, Inaba) 569B and the Tn*5*-induced perosamine biosynthesis *rfb* mutant V663 (58). *V. cholerae* O1 (classical, Inaba) cultures were grown for TCP induction as previously described (60), with the minor modification that growth was for 2 h followed by 2 to 4 h of shaking at 180 rpm before harvesting. Non-TCP-inducing conditions corresponded to growth in Difco nutrient broth at 37°C and shaking at 200 rpm with normal aeration. Viable counts of cultures were derived by comparing estimates of broth cultures in a Neubauer chamber, after vigorous vortexing to separate clumped cells, with serial dilutions onto nonselective media and overnight aerobic incubation at 37°C.

Plasmid constructions. *E. coli* DH5α [F$^-$ *supE44* Δ*lacU169* (φ80*lacZ* ΔM15) *hsdR17 recA1 endA1 deoR gyrA96 thi-1 relA1*] was used for genetic manipulations, performed as described by Sambrook et al. (45). *tcpTEF* were cloned as a 4.8-kb *Bgl*II fragment from the previously described plasmid pPM2632 (30) into the *Bam*HI site of pBluescript SK (Stratagene) in the appropriate orientation for expression from the T7 promoter to give plasmid pPM4101. DNA sequences were confirmed by restriction analysis and by M13 dye primer sequencing on an Applied Biosystems model 373A automated sequencer, as described by the manufacturer.

Protein expression and pulse-chase labelling procedures. T7 polymerase-driven overexpression and pulse labelling with [^{35}S]methionine were performed as previously described (55) in *E. coli* DH5α. The plasmids pGP1-2 (encoding the T7 RNA polymerase) and pPM4101 were introduced into *V. cholerae* JBK70, and pulse-labelling was performed as previously described (20), except that initial growth was performed in *tcp*-inducing conditions where indicated. After exposure of the [^{35}S]-labelled protein to a BAS-IIIS imaging plate (Fuji Photo Co., Ltd.), densitometry was performed in a Fuji BAS-1000 phosphorimager and analyzed with the AIS software package (version 2.0, Rev. 1.5, 1995; Imaging Research, Ontario, Canada) by density-by-area summation for individual bands.

Immunofluorescent techniques. The method used was a minor modification of one previously published (21). Round glass coverslips were boiled for 1 min in 0.1 M HCl before storage in 95% ethanol prior to air-drying for use. Poly-L-lysine (100 μl, 0.1 mg/ml) was pipetted onto coverslips placed in a 24-well flat-bottomed tissue culture tray (Costar) and incubated at room temperature for 5 min before being washed with PBS. Cells were prepared in 30% sucrose in phosphate-buffered saline (PBS) (pH 7.4) with or without 3 mM EDTA (pH 7.4) at approximately 5×10^7 CFU/ml and held on ice for 10 min. One hundred microliters of the cell suspension was pipetted gently over the coverslip and 400 μl of sterile PBS was added at 25°C before centrifugation at 500 rpm for 10 min. The wells were aspirated dry and 300 μl of 2% paraformaldehyde–0.1% glutaraldehyde was added for a further 10 min. After three gentle washes with PBS, 1% (wt/vol) fetal calf serum (FCS) in PBS was added in a 10-min blocking step before replacement with the primary antibody in 10% FCS in PBS. After 90 min of covering with a moistened paper towel, a further three PBS washes were followed by the addition of secondary antibody in the same manner for 30 min. After a further three washes as above, with the last held for 5 min, coverslips were gently aspirated dry and mounted upside down on a clean glass microscope slide with a 25-μl drop of 1% glycerine–PBS before sealing with acrylic nail varnish. All procedures after fixation were performed at room temperature (20 to 25°C). The primary antibody was Tc20.2 at a 1:10 dilution, and the secondary antibody was Texas Red-conjugated polyclonal goat anti-mouse (Pierce Chemicals) used at a 1:100 dilution.

Transmission electron microscopy and cryosectioning. The ultrathin cryosectioning procedures were slightly modified from a published method (10). Cells were washed and resuspended in 30% sucrose–PBS, pH 7.4, and held for 10 min in a 1.5-ml Eppendorf microcentrifuge tube, before pelleting and resuspending in 6.7 M (ca. 70%) sucrose–1% agarose–PBS, cooled to 45°C prior to use. Embedded cells were cooled at 4°C before being cut from the agarose and kept in 2.3 M (ca. 70% [wt/vol]) sucrose–PBS (for 2 h) before sectioning. Sections were cut at −100°C in a Reichert Ultracut S cryomicrotome, and the specimens were mounted directly on Ni grids (200 mesh, Graticules) for examination in a Philips CM100 transmission electron microscope. Ten-nanometer gold particles conjugated to protein A or goat anti-mouse sera (Amersham) were used to detect bound primary antibodies.

Physical fractionation methods: subcellular fractionation and flotation gradients and protein electrophoresis and immunoblotting. Fractionation of *V. cholerae* cultures was performed either by direct disruption of PBS-washed cells in a French pressure cell for loading onto density gradients or as follows: cells were pelleted at 7,000 × *g* from culture and resuspended (ca. 10^{12} CFU/ml) in 20% (wt/vol) sucrose–30 mM Tris-HCl, pH 8.0, followed by addition of lysozyme and EDTA, pH 8.0 (to final concentrations of 100 μg/ml and 10 mM, respectively). The periplasm was taken as the supernatant after a further 20,000 × *g* centrifugation for 10 min. The pellets were resuspended in 3 mM EDTA, pH 7.3 (ca. 3×10^{11} CFU/ml), before lysis in a French pressure cell. Unlysed cells were pelleted at 7,000 × *g* for 10 min, and final centrifugation was performed at 90,000 × *g* for 60 min to separate membrane and cytoplasmic fractions. All samples were stored at −20°C after phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1.0 mM. Protein concentrations of samples were determined with the BCA protein estimation kit (Pierce Chemicals).

Discontinuous sucrose density gradients were created in 10-ml Beckman Ultra-Clear polyallomer tubes with 1-ml layers (each) of 60, 56, 52, 48, 44, 40, 36, 32, 28, and 24% (wt/wt) sucrose–PBS, and the specimen was underloaded in 60% sucrose–PBS. Centrifugation was performed in a Beckman SW41Ti swing-out rotor for 48 h at 220,000 × *g*. Aliquots of 500 to 700 μl were collected from the bottom of the tube through a large bore needle and immediately frozen at −20°C for further analysis.

The harvesting of outer membrane blebs using LiAc-LiCl was performed essentially as previously described (33). Blebbing of the outer membrane was induced by aerobic shaking incubation for 1 h at 42°C in LiCl (200 mM)-LiAc (100 mM)-EDTA (10 mM), pH 6.0. Lysed cells and large membrane fragments were pelleted (20,000 × *g*, 30 min) before collection of outer membrane blebs from the supernatant (100,000 × *g*, 60 min). Pellets from the initial (20,000 × *g*) and from the second (100,000 × *g*) centrifugation steps were loaded in equal protein amounts and run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before staining with Coomassie brilliant blue G250 or transfer to nitrocellulose for immunoblotting.

SDS-PAGE was performed in 15% polyacrylamide under constant voltage conditions (180 V) but otherwise according to Lugtenberg et al. (25). Western transfer and immunoblotting were performed in the usual manner (58) with either anti-569B-165 (αTcpA) serum at 1:2,000 or the Tc20.2 monoclonal antibody (αTcpA) at a 1:100 final dilution. Antisera to TcpT were used at 1:2,000. Detection of immunoblots was performed by using either alkaline phosphatase-labelled goat anti-rabbit antibody, with addition of nitroblue toluidine (NBT) and bromo-chloro-indolylphosphate (BCIP) as described previously (45), or by using horseradish peroxidase-labelled goat anti-rabbit antibody and the enhanced chemiluminescence (ECL) detection kit according to the manufacturer's instructions (Boehringer-Mannheim).

Salt and detergent extraction from membranes. Membrane fractions derived by centrifugation at 90,000 × *g*, after washing and removal of unlysed cells as described above, were resuspended in PBS containing either 1.0 M NaCl, 6.0 M urea, 2% Triton X-100, or 1% Sarkosyl (no $MgCl_2$). NaCl elution was performed on ice, while urea and detergent extractions were performed at 30°C, all for 45 min. Pellets obtained at 90,000 × *g* were compared with equal volumes of supernatant, run on SDS-PAGE, and immunoblotted for TcpA.

Chemical cross-linking. In a slight modification of a published method (47), cells from 569B or V663 cultures were washed and resuspended in 50 μl of buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.8], 140 mM KCl, 10 mM NaCl, 2.5 mM $MgCl_2$) at ca. 5×10^7 CFU/ml and mixed with 2 or 4 μl of freshly prepared solution of 5 mM DSP in dimethyl sulfoxide. Cross-linking was performed on ice for 30 min and quenched with L-lysine (10 mM final concentration). DSP was prepared fresh on each occasion in dimethyl sulfoxide. Treated specimens were subsequently run on SDS-PAGE in the presence or absence of reducing conditions (boiling for 3 min with or without β-mercaptoethanol, respectively).

Protease accessibility. These experiments were performed according to a published procedure (23), with minor modifications. Whole cells grown for TCP induction were resuspended in PBS–30% sucrose with or without 5 mM EDTA and held for 30 min on ice. Trypsin (at 100 and 500 μg/ml) or proteinase K (at 10, 50, and 100 μg/ml) was added after washing of cells in PBS-sucrose, and samples were kept on ice for a further 30 min before pelleting and resuspension in sample buffer. Samples were stored at −20°C before electrophoresis as described above.

Fucose-resistant hemagglutination. Cells grown under TCP-inducing conditions were pelleted, washed, and resuspended directly in PBS at ca. 2×10^{11} CFU/ml before lysis in a French pressure cell. Fresh murine erythrocytes, obtained by retro-orbital bleeding of anesthetized adult BALB/c mice, were washed and resuspended at 1:200 dilution (packed cell volume) in a modified KRT buffer solution (0.13 M NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 2.7 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4, 10 mM *N*-[2-acetamido]-2-iminodiacetic acid [ADA], pH 7.0) with 1% (wt/vol) L-fucose for use in the assay. Lysates were stored at −20°C and were loaded in equal amounts of total protein, as estimated by the BCA method. Serial twofold dilutions in the same fucose-containing buffer were performed by multichannel pipette in a 96-well round-bottomed microtiter tray (Costar). Forty microliters of lysate or cell suspension was serially diluted and mixed with 40 μl of erythrocyte suspension, and the results were read after 2 h of standing at room temperature.

RESULTS

EDTA pretreatment promotes antibody labelling of TcpA in V663. Characterization of the avirulence phenotype of *rfb* mutants of *V. cholerae* has identified a defect in TCP assembly and apparent accumulation of processed subunit in the membrane fraction and periplasm. Surface labelling of subunit could not be demonstrated by immune electron microscopy (IEM) in these strains but was restored, along with the smooth LPS phenotype, upon complementation by a 20.1-kb *Sac*I fragment encoding the *rfb* region (16). In order to examine this assembly defect in more detail, a monoclonal antibody specific for the pilin subunit (TcpA) has been used in immunofluorescence (IF) studies. V663 (*rfb*::Tn*5*), and the isogenic parent strain 569B, were grown under TCP-inducing conditions and prepared identically for IF labelling of whole cells either with or without prior exposure to EDTA. The cells were examined by phase contrast before switching to immunofluorescence, so that the fluorescent cells could be clearly identified. Three representative fields were examined for each specimen, and results were found to be consistent on separate occasions (Fig. 1). Approximately 60 to 70% of V663 whole cells were labelled after EDTA treatment, compared with significantly fewer (10 to 30%) untreated V663 (Fig. 1). In the *wt* parent, external pilin bundles were labelled. These were detected in the medium as well as in association with cells, identical to findings in IEM studies in this laboratory and elsewhere (14, 56, 60), but there was no labelling of *wt* cells themselves even in the presence of EDTA. All cultures were subjected to viable counts, with 569B being 100% viable and V663 cells being >75% viable under the growth conditions described.

Anomalous distribution of TcpA subunit in flotation gradients. The significantly greater amount of cell surface binding of antibody in immunofluorescent studies after EDTA pretreatment suggests that TcpA within the outer membrane or periplasm is made accessible by outer membrane disruption. In order to better define the apparent membrane association, whole cell lysates were underloaded into a sucrose flotation gradient. The locations of major outer membrane proteins and the cytoplasmic membrane-associated TcpT (by immunoblot) are indicated for comparison with the sucrose density of fractions (Fig. 2). There is some predominance of the TcpA pilin subunit in outer membrane protein-containing fractions of V663, but it is seen to distribute widely throughout the gradients, with nearly identical distribution for the *wt* parent (Fig. 2). This distribution is mirrored in the classical *wt* strain Z17561 and in isogenic export mutants in which TcpA is cytoplasmic membrane associated (15), suggesting that TcpA behaves irregularly on sucrose gradients. Thus, while TcpA appears to fractionate to the periplasm (in V663 but not the *wt* parent) by simple differential centrifugation of cell lysates (16), and while other type 4 pili form discrete bands on sucrose gradients (5, 35, 63), there is no difference detectable between our samples in the density-based flotation gradient method.

Outer membrane blebs contain TcpA in both *wt* and *rfb* strains. Because of anomolous flotation in density gradients, it was necessary to use a number of other approaches to derive information about membrane localization. Outer membrane material was prepared from whole cells of 569B and V663 by LiAc-LiCl extraction (33). Outer membrane material harvested by the second centrifugation (100,000 $\times$ g) made up approximately 1% of the total protein content (estimated by the Bradford method) derived from the first step (20,000 $\times$ g) for both 569B and V663. The TcpA subunit was present in outer membrane blebs harvested from V663 in proportions at least equal to its representation in residual whole cells, as judged by immunoblotting (Fig. 3). Despite the absence of TcpA from membrane blebs in isogenic *tcpT* and *tcpT rfb* mutants, which have a cytoplasmic membrane export block (15), this experiment does not differentiate between a periplasmic and an outer membrane pool in V663, confirming only that TcpA is released with outer membrane material from these cells. Furthermore, the anomalous flotation in density gradients and the hydrophobic nature of the bundle-forming pilin meant that it was not possible to rule out a weakly associated inner membrane or periplasmic pool fractionating nonspecifically with the outer membrane.

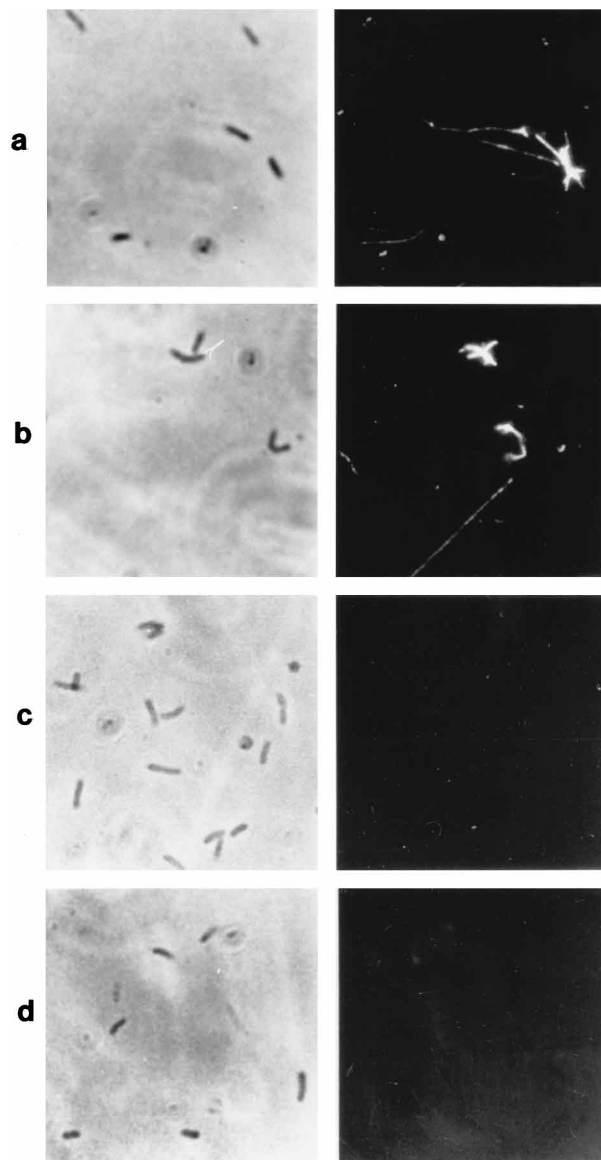

FIG. 1. IF microscopy of 569B and V663 whole cells. Matching phase-contrast (left panels) and immunofluorescent (right panels) images of *wt* (569B) cells show labelling of TCP bundles without (a) and with (b) prior EDTA treatment. Approximately 10 to 30% of V663 cells are labelled without EDTA pretreatment (c), while more than 70% are labelled when pretreated with EDTA (5 mM) (d). Tc20.2 (1:10) IgG monoclonal antibody and Texas Red conjugated goat anti-mouse antisera (1:100) were used to label TcpA.

TcpA localization in ultrathin cryosections of *rfb* mutants. Cells taken directly from culture, plasmolysed in PBS-sucrose, and sectioned at −100°C suffer minimal disruption to native

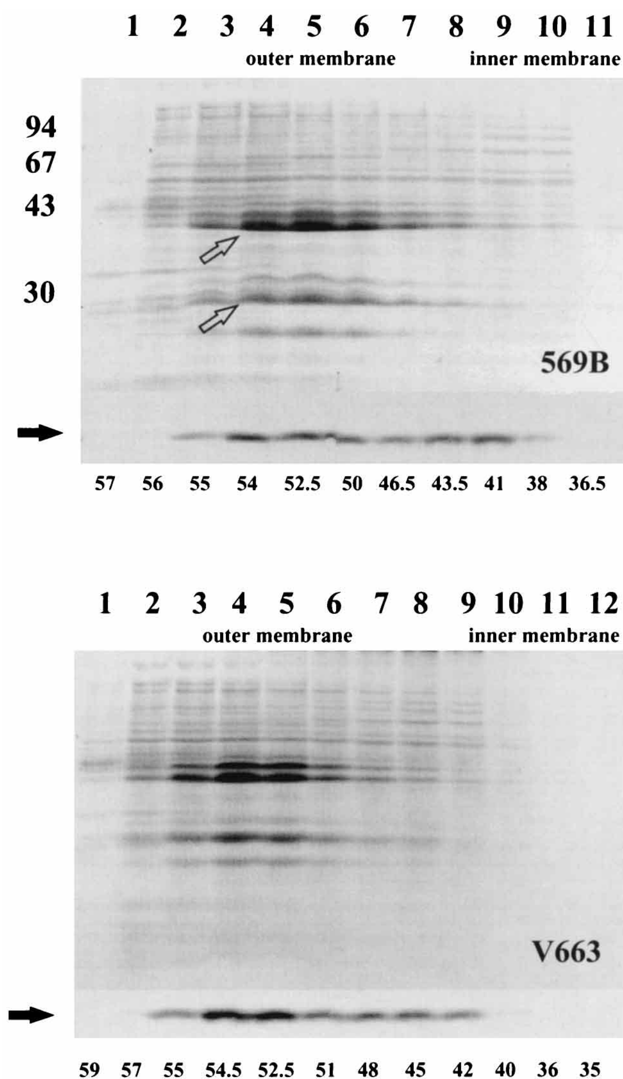

FIG. 2. Distribution of the TcpA subunit in sucrose density gradients. 569B (upper panel) and V663 (lower panel) fractions in sucrose flotation gradients are shown. TcpA immunoblots (solid arrows) are presented under Coomassie-stained SDS-PAGE gel in each panel. Equal volumes of each fraction were loaded, and sucrose densities are given (% [wt/wt]) under every lane. Size markers (in kilodaltons, at left) and the location of the major outer membrane proteins of 569B (6) are indicated (open arrows). TcpT, an inner membrane protein (15), was detected in fractions 9 to 11 and 10 to 12, respectively, of 569B and V663 (not shown).

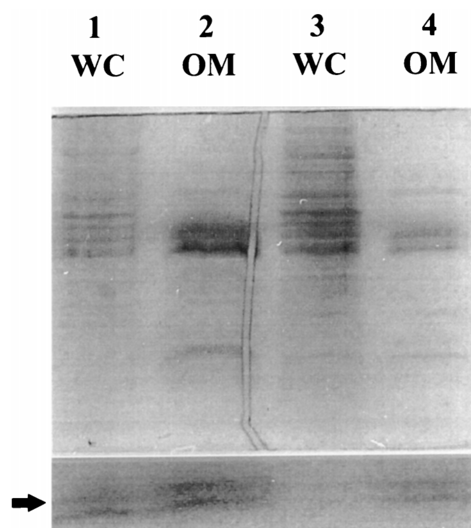

FIG. 3. LiAc-LiCl-EDTA-extracted outer membrane material from V663 contains TcpA. Coomassie-stained SDS-PAGE gel and the corresponding immunoblot (lower panel), showing TcpA (arrow), are presented. The whole-cell and large membrane fragments (lanes WC, collected at 20,000 × *g*) and the outer membrane material (lanes OM, harvested from the resulting supernatant at 100,000 × *g*) are shown for 569B (lanes 1 and 2) and V663 (lanes 3 and 4). Samples were loaded in approximately equal protein amounts, and the immunoblot was developed by detection of bound α569B-165 polyclonal anti-TcpA sera by the NBT-BCIP colorimetric method.

structures and antigens and are relatively free of fixation artifact (10). V663 cells grown as described for TCP induction were prepared, and 800-μm sections were incubated with the specific monoclonal antibody to TcpA, followed by goat anti-mouse antibody conjugated to 10-nm gold particles. Labelling was predominant in the periplasmic space and at the inner face of the outer membrane (Fig. 4). Gold particles were most conspicuous where longitudinal poles of cells were transected but could also be seen in oblique and longitudinal sections. There was no obvious pilus structure discernible and the label appears to be relatively diffusely distributed, even in the membranes. The *wt* cells could not be labelled with the monoclonal antibody other than in external bundles (not shown).

TcpA subunit interactions within *wt* and *rfb* strains. We have shown that the TcpA subunit is associated with the outer membrane in the *rfb* mutant V663 but is not surface presented. Accumulated subunit can be found here and in the periplasmic space, and also in association with the cytoplasmic membrane, by specific labelling of ultrathin sections. It was therefore of interest to determine the influence of this export arrest on the physical characteristics and interactions of the TcpA subunit. Either of two different detergents (2% Triton X-100 and 1% Sarkosyl) efficiently extracted TcpA from the membrane fraction (Fig. 5). TcpA was eluted in 6.0 M urea but not in 1.0 M NaCl. The detergent extraction characteristics are consistent with hydrophobic subunit interactions with other subunits and/or membrane elements. TCP fibers centrifuged out with membrane fractions in the isogenic *wt* preparations were identically extracted and eluted in parallel, suggesting strongly that subunit-subunit interactions rather than true membrane associations determine the behavior of TcpA under these conditions and that interactions of TcpA in the fully assembled fiber cannot be distinguished from those within the assembly-defective strain V663 by these criteria.

Identical near-neighbor relationships of subunits in *wt* and *rfb* strains. Since it was apparent that pilin subunits in the membrane fraction of V663 were firmly associated, it was important to look for evidence of fiber assembly in a different manner. Membrane-permeable (DSP) and water-soluble (DTSSP) cross-linkers with reducible disulfide bonds were used to explore near-neighbor relationships of TcpA subunits. Whole cells and gradient fractions from regions corresponding to inner and outer membranes were cross-linked in order to detect differences in multimerization of TcpA. These agents span up to 11 Å between cross-linkable residues with reactive amine groups (e.g., lysyl and aspartyl) and gave reproducible and consistent results. An identical pattern of ascending multimers was found in all specimens in which cross-links were not reduced and in whole-cell or gradient fractions of either V663 or 569B. TcpA migrated at the same molecular weight in reducing and nonreducing conditions in all samples, and

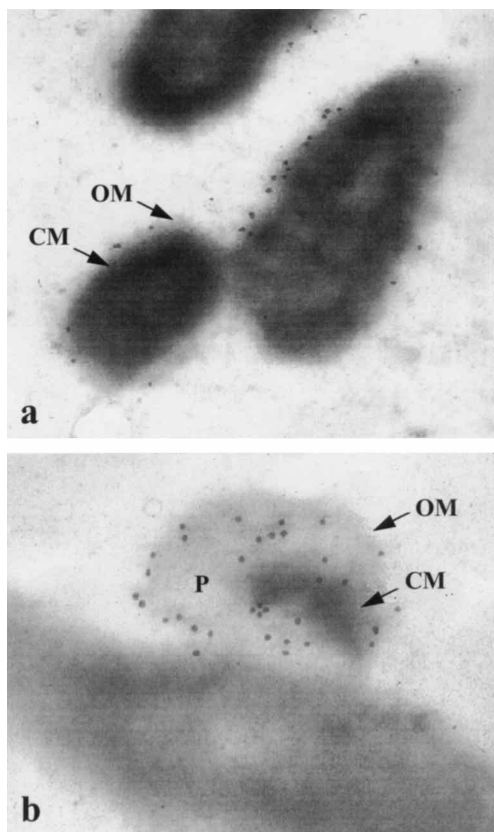

FIG. 4. Ultrathin cryosections of V663 reveal TcpA in both cytoplasmic (CM) and outer membranes (OM). A section apparently through the longitudinal pole of a cell with more marked plasmolysis (b) clearly illustrates gold particles in the periplasmic space (P). Bound Tc20.2 monoclonal antibodies were detected by goat anti-mouse antibodies conjugated to 10-nm gold particles.

there were no multimers evident in the absence of cross-linker (Fig. 6).

Protease accessibility and fucose-resistant hemagglutination suggest mature secondary structure in translocation-arrested subunits. Whole cells prepared as for IF, but without fixation, were exposed to proteinase K (10, 50, and 100 μg/ml)

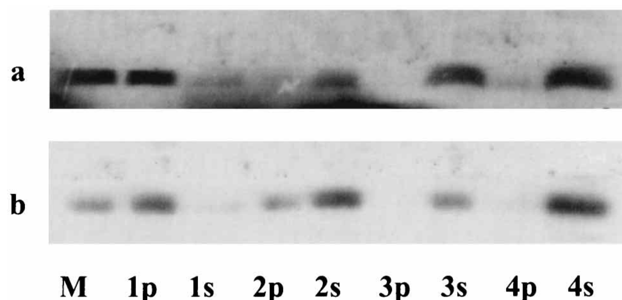

FIG. 5. Salt and detergent extraction of TcpA from membranes of 569B (a) and V663 (b). After extraction, paired samples of pellets (p) and supernatants (s) were obtained by centrifugation at 90,000 × *g* for 1 h. Pellets were resuspended in the original volume, equal to that of supernatant, and samples were run in equal volumes on SDS-PAGE gel for the TcpA immunoblot. Lanes contain untreated membrane (M) or membrane pellet after treatment with either NaCl (1.0 M), 30 min on ice (lanes 1); urea (6.0 M), 30 min at 30°C (lanes 2); 2% Triton X-100, 30 min at 30°C (lanes 3); or 1% sodium lauryl sarcosinate (Sarkosyl), 30 min at 30°C (lanes 4).

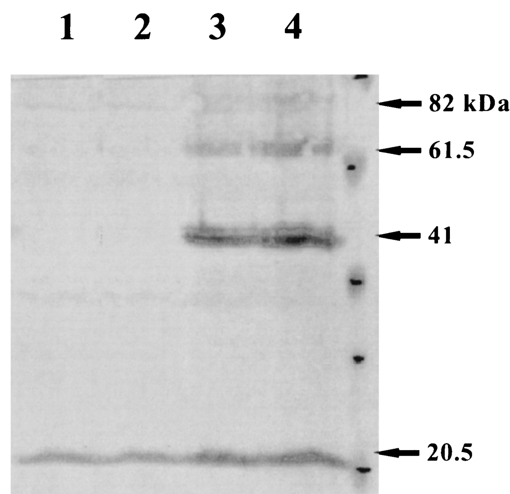

FIG. 6. Chemical cross-linking of the TcpA subunit in V663 is indistinguishable from that of TcpA subunit within *wt* TCP. DSP-treated whole cells of 569B (lanes 1 and 3) and V663 (lanes 2 and 4) were run in both reducing (lanes 1 and 2) and nonreducing (lanes 3 and 4) conditions. Sizes are indicated in kilodaltons.

and trypsin (100 and 500 μg/ml) in an attempt to probe differences in protease accessibility in the presence and absence of EDTA. TcpA in both 569B and V663 was degraded no more readily than any of the major outer membrane proteins at high concentrations of proteinase K, consistent with the previously demonstrated incomplete susceptibility of TcpA to proteases (54), and no differently in the presence of EDTA pretreatment and washing, as for IF samples. Since identically prepared EDTA-pretreated cells can be labelled by monospecific antisera to TcpA in IF experiments, it is unlikely that inaccessibility to antigen explains this result. This suggests that the translocation-arrested TcpA subunit in V663 is no more susceptible to these proteases under the conditions described than TcpA within the assembled pilus of the *wt* isogenic parent strain 569B.

Concentrated whole-cell lysates of 569B and V663 were tested for fucose-resistant hemagglutination of fresh murine (adult BALB/c) erythrocytes as described for TCP (57). While the method was relatively insensitive in our hands (requiring ca. 10^9 CFU of *wt* 569B per ml to agglutinate 0.5% erythrocytes in PBS or in the buffer described in 2 h at room temperature), it discriminated clearly between TcpA-negative and TcpA-positive lysates. Assays were repeated on several occasions, and TcpA expression was confirmed by immunoblot. Unmodified TcpA released from V663 by mechanical lysis alone is competent for fucose-resistant hemagglutination (Table 1). Significant reduction of titers by specific monoclonal antibody confirms that this is a TcpA-specific phenomenon, as previously demonstrated (38, 56). Incomplete blocking by this monoclonal antibody may be explained by the large amounts of antigen present, since a greater than 1:10 dilution gives poor labelling in IEM studies (13a, 17a).

Potential outer membrane elements involved in the assembly of TCP. Having established that the outer membrane was the likely barrier to passage of the TcpA subunit, our attention turned to possible elements that might be disrupted as a consequence of the *rfb* mutation. The complementing fragment for the TCP defect of V663 is internal to the known *rfb* region, containing only O-antigen biosynthesis genes and no likely candidates for a direct transport role for TCP (29). Both *tcpC* and *tcpF* are members of the operon dedicated to TCP assem-

TABLE 1. Testing of whole-cell lysates for fucose-resistant hemagglutination[a]

Cells	Hemagglutination titers (mean)[b]			
	569B		V663	
	AKI	NB	AKI	NB
Unlysed whole cells	32, 64, 64 (53)	2, 4, 4 (3.3)	2, 4, 2 (2.7)	2, 2, 2 (2)
Whole-cell lysates	32, 64, 64 (53)	ND	32, 64, 32 (42.7)	ND
Whole-cell lysates + MAb	8, 32, 16 (18.7)	ND	8, 8, 16 (10.7)	ND

[a] Whole cells and lysates were adjusted to ca. 2×10^{11} CFU/ml in the starting material. Cultures were grown under optimal TCP-inducing conditions (AKI) or in non-inducing conditions (NB), as indicated.

[b] The results of three experiments are recorded as reciprocals of the highest dilution in which a mat of erythrocytes, rather than a button of non-cross-linked cells, was visible at the bottom of the wells. Serial dilutions are twofold and are recorded as the final dilution (addition of neat sample is therefore a 1:2 final dilution and is recorded as 2). The monoclonal antibody Tc20.2 (MAb) was used at a final dilution of 1:10. Cell numbers were estimated in a counting chamber before lysis. Total protein content was also calculated (BCA method) to confirm equivalence of final samples before use.

bly and encode a lipoprotein (29, 37) and a potential porin-like outer membrane protein, respectively (20, 30).

It has been observed that *tcpF* in a range of constructs under the influence of the induced *trp/lac* promoter in pBluescript is stable in *E. coli* but cannot be introduced into *V. cholerae* strains, including 569B. These were tolerated in the opposite orientation, however, in which expression would be subject to the uninduced T7 promoter, and identical constructs in which *tcpF* was partially deleted were readily moved into *V. cholerae* strains (unpublished observations). Introduction of pGP1-2 into *V. cholerae* JBK70, as has been previously described (20), permitted overexpression of TcpF from pPM4101 in the *V. cholerae* background. The presence of a kanamycin resistance gene in both the V663 chromosome and the pGP1-2 plasmid encoding T7 RNA polymerase (53) and the requirement for long labelling times (20 min) due to relatively poor labelling in *V. cholerae* (e.g., strain JBK70) obstructed attempts to perform pulse-chase experiments of TcpF in V663. JBK70/pGP1-2 carrying plasmid pPM4101 (*tcpTEF*) was grown either in Difco nutrient broth at 30°C or under the *tcp*-inducing conditions of AKI broth at 30°C and 5% CO_2 (58) before induction of T7 polymerase. TcpA was detected by immunoblot with anti-569B-165 sera (49) in whole cells grown under AKI conditions but not in those grown in nutrient broth (not shown). When expressed under control of the T7 RNA polymerase in a pulse-labelling experiment in *V. cholerae*, a lower-molecular-weight product (TcpF*) consistent with the predicted type I leader peptidase (Lep)-dependent processing of TcpF (20, 30) is relatively predominant in the setting of prior *tcp* induction. TcpF* is responsible for most (69%) of the detectable radiolabel under these conditions, as determined by density-by-area summation of [^{35}S]methionine-labelled bands in a phosphorimager. The differential between TcpF (F) and TcpF* (F*) is less striking in the absence of prior *tcp* induction (Fig. 7). While trivial explanations such as differences in growth rates and different expression efficiencies in titrating Lep activity cannot be discounted, it suggests the possibility of an effect of localization on stability, especially in view of the apparent toxicity of the gene product in *V. cholerae.*

Like V663, *E. coli* DH5α has no O antigen and, also like V663, requires only the presence of the complementing *rfb* region to express a full *V. cholerae* O1 Inaba LPS (16). TcpF has been shown previously to be cleaved in *E. coli* and *V. cholerae* (20, 30). Thus, when TcpF is overexpressed as [^{35}S]methionine-labelled protein in either background, a band at 38 kDa corresponding to the *tcpF* product is accompanied by a second band approximately 1.5 kDa smaller (Fig. 7 and 8). However, this processed form (TcpF*) can be seen to disappear in a pulse-chase experiment in the *E. coli* background, while unprocessed TcpF persists (Fig. 8). The apparent instability of TcpF* after processing suggests failure to correctly localize and/or associate with other proteins in the normal manner. Unprocessed TcpF might be predicted to accumulate in the cytoplasm as hydrophobic aggregates, perhaps casually associated with the inner membrane. It is possibly relevant that TcpE, predicted to be an integral inner membrane protein which complexes with other products of the *tcp* operon in vivo (20, 30), is similarly unstable in comparison to TcpF.

DISCUSSION

Previously described *rfb* mutants of classical *V. cholerae* 569B that are unable to synthesize LPS O antigen due to a block in perosamine biosynthesis (62) appear to have a TcpA subunit translocation defect at the level of the outer membrane (16). The parent strain presents all detectable TcpA within the mature pilus, but there is identical and relatively uniform distribution of subunit in a flotation gradient whether or not it is exported as assembled pilin. Direct labelling of subunit in cryosections, along with the IF data and findings on simple physical fractionation, clearly shows TcpA to be accumulating in a stable configuration beyond the cytoplasmic membrane of the *rfb* mutant, V663. Although it is not surface presented, processed pilin can be identified by specific antisera in chemically extracted material and in ultrathin sections of the outer envelope.

It remains a moot point as to whether periplasmic spillover of subunit from the outer membrane is real or whether it

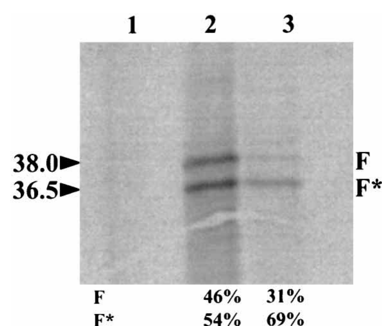

FIG. 7. In *V. cholerae* JBK70/pGP1-2/pPM4101, [^{35}S]methionine-labelled TcpF is predominantly of the lower-molecular-weight form (F*) at 15 min in the setting of prior *tcp* induction (lane 3) but not in the absence of prior TCP induction (lane 2). Lane 1 contains vector-only control. Densitometric analysis of each band is presented as a whole-number percentage of the combined total of the pair of bands, as determined by density-by-area summation after detection of radioactively labelled protein in a phosphorimager.

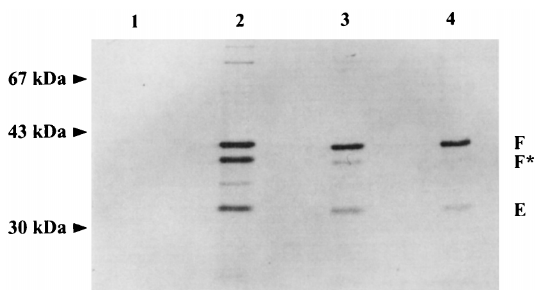

FIG. 8. The lower-molecular-weight form of TcpF (F*) disappears in a pulse-chase of [^{35}S]methionine-labelled TcpF in *E. coli* DH5α, while the higher-molecular-weight form (F) remains stable. The estimated sizes of ca. 36.5 and 38 kDa are consistent with the loss of 20 amino acids in the predicted leader peptide. Lanes 1 to 4, samples at *t* = 0, 2, 10, and 60 min, respectively, after labelling (excess unlabelled methionine added at 1 min). TcpE (E), predicted to be an integral inner membrane protein, is also seen to disappear at a similar rate.

simply reflects the loose association of translocation-arrested subunits. These might be predicted to cofractionate with the membranes in any case, and the periplasmic pool seen in ultrathin cryosections could simply be a consequence of loss into the (otherwise only potential) space created by plasmolysis for cryosectioning. Unprocessed type 4 pilin subunits from *P. aeruginosa* stably associate with the *E. coli* cytoplasmic membrane when overexpressed (35), although they are clearly not entrained in an export or assembly system. Careful studies of pilin pools in *P. aeruginosa* PAK and of F pili in *E. coli* suggest that the pilin subunit represents no more than about 5% of the protein mass of the cytoplasmic membrane and less in the outer membrane (28, 63). Periplasmic pools have not been described for strains of type 4 piliated organisms, although PhoA$^+$ transcriptional fusions of *N. gonorrhoeae* MS11 type 4 pilin subunits are stable in the cytoplasmic membrane after processing in *E. coli* (4), as are similar fusions of PAK pili in *P. aeruginosa* (53). The extent to which such data reflect actual membrane assembly pools and processes is difficult to determine, since chemical and physical methods employed in the fractionation process may distort the natural distribution of pilin. While failure to identify significant periplasmic TcpA in cryosections of the *wt* parent strain is consistent with other data described here and published elsewhere (4, 16), it does not exclude an important periplasmic stage in the normal assembly process. One should also be mindful that the bundle-forming TCP of *V. cholerae* may be more likely to associate with other hydrophobic elements than other type 4 pili, which are found in a relatively discrete location in sucrose density gradients (5, 35, 63).

It has not been possible to determine the precise assembly state of TcpA subunits prior to their presentation in a complete external fiber from the data presented here, but a number of other lines of evidence suggest that simple conformationally directed self-association is the mechanism by which the fiber is formed. The C-terminal disulfide loop region of the type 4 pili (from *V. cholerae* [TCP] and from *P. aeruginosa*) contains the essential functional regions for adhesion (6, 54), while the highly conserved N-terminal region is critical for assembly (36). Normal folding of the TcpA subunit in the periplasm of *V. cholerae* is dependent upon periplasmic disulfide oxidoreductase activity, and loss of this activity results in the assembly of morphologically normal but biologically dysfunctional pili (as measured by loss of adhesion in vivo and loss of specific TcpA-mediated hemagglutination in vitro) (38). In addition, surface assembly of similarly dysfunctional pili lacking normal C-terminal disulfide loops in *P. aeruginosa* (6) and models derived from the recently solved crystal structure of the type 4 pilin of *N. gonorrhoeae* MS11 (36) also suggest that subunits are capable of packing into a multimer after processing at the cytoplasmic membrane. The hemagglutination and protease susceptibility data are consistent with normal folding of the subunit C-terminal globular head domain in V663 and further imply that TcpA in V663 has achieved normal secondary structure in the periplasm.

The apparent density of TcpA subunits in a flotation gradient and their detergent solubility, salt elution characteristics, and ability to be chemically cross-linked do not differ between the *wt* and the *rfb* mutant, as determined by the methods used. The identical behavior of the TcpA subunits suggests that these characteristics reflect subunit-subunit rather than subunit-membrane interactions. The requirement for strong salt conditions to elute the subunit from membrane fractions is consistent with the predicted interaction centered on the +5 glutamate residue absolutely conserved in type 4 pilin and pilin subunit-like proteins and probably critical for correct assembly (36, 41). Spontaneous formation of trimers and higher-order multimers from disaggregated or incompletely assembled TcpA in vitro cannot be excluded. However, previously published data show that while purified type 4 pili solubilized in β-octyl-glucoside may form high-molecular-weight aggregates which can be reduced to minimally surface-hydrophilic dimers at pH 10.8 (35a), monomeric assembly is the only model consistent with the known crystal structure of type 4 pili (36). Cells taken from culture for cross-linking experiments reported here are exposed only to a previously described detergent-free physiological buffer (47), and the assembly process of type 4 pili appears to be critically determined by the highly conserved N-terminal region to whose structure all subunits, including TcpA, conform strictly (13, 14, 36, 56).

The 18-kDa type 4 pilin subunit of *P. aeruginosa* PAK is packed in a helical array of 4 subunits per turn, of 4.1-nm pitch (7). The estimated diameter of the mature type 4 fiber from a number of species is around 6 nm (5, 8). A structure of these dimensions, or the 6.5-nm estimated diameter of filamentous phage, would need a large diameter channel (compared with the 1.1 nm of the typical trimeric porin) such as might be formed from the homomultimeric pIV protein, homologs to which are highly conserved in outer membrane transporter and type 4 pilin systems (9, 27) and which is sufficiently homologous with some of these homologs to form mixed multimers in vitro (21). Such a protein appears essential for type 4 pilin assembly in *N. gonorrhoeae* (59), and it may be that exploration of the incorporation of the unusual tip adhesin in that pilus system (43) will give us vital clues to the function of conserved outer membrane proteins in the assembly process.

There is no member of this protein superfamily encoded within the *tcp* operon. While such a protein may be encoded elsewhere, there are other candidate outer membrane proteins, TcpC and TcpF, which may fulfill this role. TcpC is a lipoprotein, processed by a type II signal peptidase, but is of unknown function (29, 37). TcpF is predicted to be an outer membrane porin-like protein, possessing a type I leader sequence and a number of possible transmembrane regions (19, 30). Tn*phoA* insertions in both of the genes lead to a TCP-negative phenotype in the classical strain O395 (57), which appears to be identical in sequence to 569B over the entire *tcp* region. Such a mutation in *tcpC* could lead to disruption of the transcriptionally linked downstream genes, but *tcpF* is immediately followed by a transcriptional termination sequence and a separate promoter for *toxT* and *tcpJ*. While neither TcpC nor TcpF has direct homologs in the type 4 pilin or general secre-

tory pathways, they could conceivably contribute to a porin-like channel permissive for macromolecular passage, as proposed for filamentous phage (44).

There are a number of examples which illustrate the importance of normal LPS to the functioning of structures in the outer membrane. Surface presentation of K99 (type 1) pili is reduced in *rfa* mutants of *E. coli*, and K99 is observed to bind LPS in vitro (39). It has been observed that rough mutants of *Shigella flexneri* fail to correctly localize the polar surface protein IcsA (46), and rough mutants of *E. coli* fail to normally assemble LamB and OmpF trimers (24, 48). An LPS-related effect may also act indirectly by affecting a product or products of the *tcp* operon that contribute directly to the stable localization of TcpF within the outer membrane, such as has been demonstrated in functionally related systems for DNA transfer (17) and protein export (11). The outer membrane disturbance occasioned by the *rfb* defect in V663 appears to be relatively subtle. The profile of proteins detectable by Coomassie staining of SDS-polyacrylamide gels differs between the mutant and its isogenic parent, but secretion of cholera toxin and DNase appears to be normal (16). It is proposed here that the apparently greater instability of TcpF* in *E. coli* may be due to incorrect localization after processing. The *E. coli* host strain lacks both O antigen and the *lacZ* protein, unlike *V. cholerae*, in which the vector *lac* promoter in pPM4101 (*tcpTEF*) is derepressed, and it is possible that incorporation of excessive amounts of the porin-like TcpF into the outer membrane explains the toxicity of *tcpF* in *V. cholerae*. Alternatively, but probably less likely, the loss of O antigen may have a more direct effect to increase the hydrophobicity of the LPS molecules, thus acting as a trap for hydrophobic TcpA subunits and preventing their assembly. A possible precedent is the inability of *N. gonorrhoeae* to correctly assemble the hydrophobic overlength L pilus (26). Resolution of these issues in TCP assembly await the definitive subcellular localization of TcpF and F* and identification of the role(s), if any, of other proteins.

In conclusion, consistent with the model derived from the MS11 type 4 pilin crystal structure (36), we propose that stable TcpA subunit associations occur after processing at the cytoplasmic membrane and are predominantly hydrophobic and easily detergent disrupted. Disturbance of normal membrane stoichiometry in O-antigenless mutants of *V. cholerae* leads to an export block at the periplasmic face of the outer membrane. The nature of the disturbance is likely to be related to an integral member of the outer membrane, and the TcpF protein is one such candidate for closer study. Whether the outer membrane participates actively in the assembly of mature fiber or is simply a barrier which must be passed remains to be determined.

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